Mechanisms of insecticide resistance in Indian malaria vector *Anopheles stephensi*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by *Cherry Lynda Dykes*.

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One sentence to describe this thesis -"It didn't go as planned but it wended its way and that's okay!" One note, which is easier said than silent—"SELF is the best competitor to compete with, & 'Good enough' is better than BEST".

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ABSTRACT

To understand the mechanisms of insecticide resistance in *Anopheles stephensi*, DDT- and pyrethroid-resistant lines were selected in the laboratory. Selection process showed fixation of L1014S-*kdr* mutation in DDT- and pyrethroid resistant colonies. Similar fixation was recorded in unselected mosquitoes (control) as well, suggesting low fitness cost of L1014S in laboratory conditions. Synergistic assays using piperonyl butoxide (PBO), a mixed function oxidase and esterase inhibitor, led to reversal of resistance in pyrethroid resistant mosquitoes but no reversal in DDT resistant mosquitoes was recorded. Elevated GST activity in laboratory strains led us to characterize the DDT resistance implicated GST epsilon genes. Quantitative-PCR showed elevated expression of AsGSTe2 and AsGSTe4 in DDT-resistant mosquitoes in both laboratory selected DDT-resistant strain and field caught mosquitoes. These observations prompted further investigations to molecularly characterize these genes examining mutational changes and the possible roles of allelic variation.

Cloning and sequencing of the full genes revealed polymorphism which resulted in four variants in AsGSTe2 and three variants in AsGSTe4. Of the four variants of AsGSTe2, two variants (AsGSTe2.1 and AsGSTe2.2) found in DDT-resistant individuals were expressed in vitro in *E.coli*. Recombinant expression and DDTase assays of AsGSTe2.1 and AsGSTe2.2 showed them to efficiently metabolise DDT. DDTase activity examined for recombinant AsGSTe4 (AsGSTe4.1 and AsGSTe4.2 and AsGSTe4.3) showed that they did not metabolise DDT. Enzyme thermostability tests showed AsGste2 variants to be highly unstable compared to the orthologues in *An. gambiae, Aedes aegypti* and its corresponding AsGSTe4 variants.

Further examination into the GST epsilon array provided evidence of tandem co-duplication of AsGSTe2 and AsGSTe4 together in the GST-epsilon array of the laboratory DDT-resistant colony. Organization of gene duplication and breakpoints in the DDT-resistant strain were identified, where at least five tandem repeats of a 3.7 kb long unit consisting of a complete AsGSTe2, e2-pseudogene and AsGSTe4 intercepted by a highly conserved 2.4 kb of insert region from an unrelated part of the genome were recorded. Polymorphism in two AsGSTe2 and three AsGSTe4 paralogs

due to non-synonymous changes beside high divergence in intervening e2-pseudogene indicated that gene duplication is not a recent event. Despite divergence in paralogs of AsGSTe2 and AsGSTe4, all genes expressed as mRNA. The copy number variation at genomic level observed in resistant individuals against the susceptible strain suggests that gene duplication may be associated with DDT resistance in *An. stephensi.*

The voltage gated sodium channel (VGSC) gene, a target site for DDT and pyrethroids was also investigated for mutational and post transcriptional changes in DDT selected and unselected *An. stephensi* lines. Two novel non-synonymous mutations Q695R and E1235Q were identified besides the classic L1014F/S *kdr* mutations. The novel mutations were found only in the susceptible phenotype which harboured the L1014. Cloning and sequencing of coding VGSC revealed 16 alternative splicing events which include exon skipping, alternative acceptor sites, mutually exclusive exons and an intron retention. While cloning experiments showed skipping of Exon 5, 2 and partial splicing of exons 18 and 24 occurring in greater numbers in the DDT resistant strain compared to the susceptible, real time analysis did not support the results. RNA editing at L1014 residue, as reported by previous investigators was ruled out in *An. stephensi.* The study outlined the importance of genetic mechanisms in resistance and provides a plausible explanation that the high expression of AsGSTe2 and duplication of the gene are linked and are responsible for the high DDT resistance in *An.stephensi.*

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LIST OF ABBREVIATIONS

Ace	acetylcholinesterase gene
AChE	acetylcholinesterase
An.	*Anopheles*
BLAST	basic local alignment and search tool
BgNaV	Blattella germanica voltage sodium channel
CDNB	1-chloro-2,4-dinitrobenzene
CHP	cumene hydroperoxide
CM	corrected Mortality
DCNB	1,2- Dichloro -4- nitrobenzene
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DEF	sss- tributylphosphothiorate
DEM	diethyl maleate
DmNaV	Drosophila melanogaster voltage sodium channel
gDNA	genomic DNA
GSH	L-glutathione
GST	glutathione *S*-transferase
HCH	Hexachlorocyclohexane
ICMR	Indian Council of Medical Research
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
IRS	indoor residual spraying
IS	Insert segment

ITMN	insecticide treated mosquito net
IVCC	Innovative Vector Control Consortium
kdr	knockdown resistance
LD	linkage disequilibrium
LLIN	long lasting insecticidal net
LSTM	Liverpool School of Tropical Medicine
LT	lethal time
MFO	Multi-functional oxidases
NGS	next generation sequencing
NIMR	National Institute of Malaria Research
NVBDCP	National Vector Borne Disease Control Programme
OC	Organochlorine
OP	organophosphate
P450	cytochrome P450
PBO	piperonyl butoxide
PCR	polymerase chain reaction
Rdl	resistance to dieldrin
RFLP	restriction fragment length polymorphism
RNAseq	RNA sequencing
s.l.	*sensu lato*
s.s.	*sensu stricto*
SNP	single nucleotide polymorphism
VGSC	Voltage gated sodium channel
WHO	World Health Organisation
WHOPES	WHO pesticide evaluation scheme
XGAL	bromo-chloro-indolyl-galactopyranoside

Chapter 1. BACKGROUND

1.1 Malaria – a major health burden

Malaria was introduced in English literature by writer Holace Walpole in 1740 to describe the disease “Mal’ aria” which in Italian means ‘foul air’ (Ernst Hempelmann, 2013) and John MacCulloch introduced it into the English scientific literature in 1827 (Chwatt 1977, pp.156-165), which later, is shortened to "malaria" in the 20th century. Malaria is caused by a protozoan parasite and transmitted from one human host to another through the bite of an infected female anopheles mosquito. The disease is prevalent in tropical and subtropical regions of the Americas, Asia and Africa. Africa accounts for most global cases of malaria (90%), followed by the South-East Asia Region (7%) and the Eastern Mediterranean Region (2%). Democratic Republic of the Congo and Nigeria account for about 40% of estimated mortality worldwide (World malaria report 2014). Figure 1.1 shows the current global malaria transmission with Africa, India and parts of South America each covering a large part of the disease burden. Recent estimates by the World Health Organization (WHO) (December 2015), showed a worldwide occurrence of 438,000 malaria deaths (range 236 000–635 000) and an estimate of 214 million new malaria cases. Considering the projected growth of the world’s population by 2030, the prediction is that more people will be residing in countries where malaria poses a risk, putting further strains on health systems and national malaria programme budgets (WHO, 2015).

India contributes nearly 80% of the cases in the Southeast-Asian region (Dev, 2013), with the large population (second highest in the world) living with the risk of malaria. In 2013, India reported 880,000 microscopically confirmed malaria cases showing comparable numbers in *Plasmodium falciparum* and *Plasmodium vivax* cases, while 1,100,000 cases with 561 deaths were recorded in 2014 by the National Vector Borne Disease Control Programme (NVDCP).

Dhingra estimated a ~13-fold underestimation of malaria-related mortality (Dhingra *et al*., 2010) and Hay suggested that malaria incidence is between 9 to 50 times greater than that reported (Hay *et al*., 2010). About 95% of the population in India resides in malaria endemic areas, where 80% of malaria reported is confined to 20% of the population residing in tribal, hilly and inaccessible areas (Gupta, 2014). The highest

incidence with 75,000,000 cases and 880,000 deaths per year was in 1947 (Shiv Lal, 2005; Das *et al*., 2012). Figure 1.2 illustrates the overall endemicity of the disease in India from the year 2001 through 2014, with the highest deaths occurring between 2006 and 2007. The launching of the National Malaria Control Programme (NMCP) in 1953 brought in a significant decline in the number of cases. Lack of a stratified system led to resurgence of malaria (~ 6.45 million cases) in India in 1976 (Sharma *et al*., 1996).

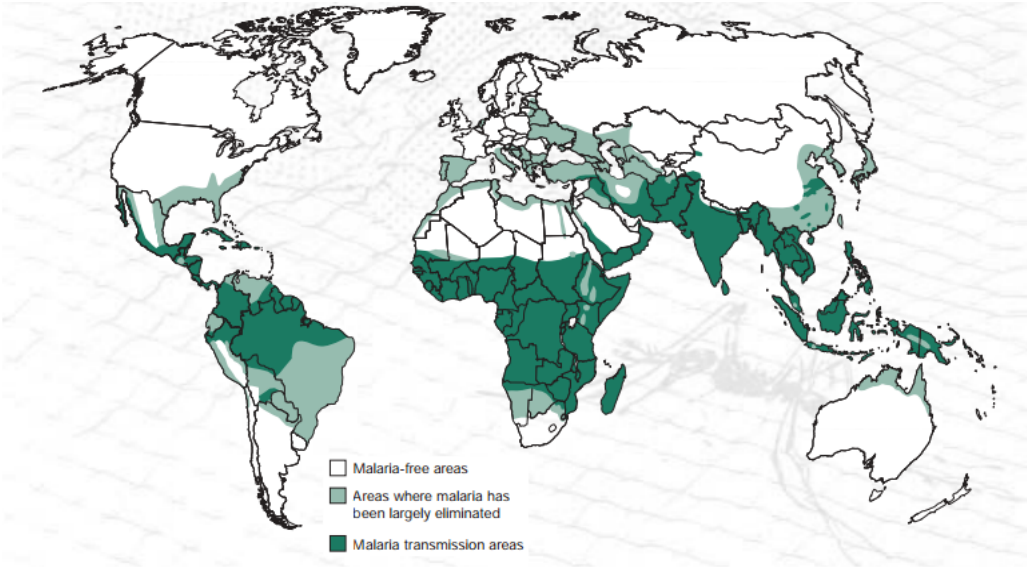
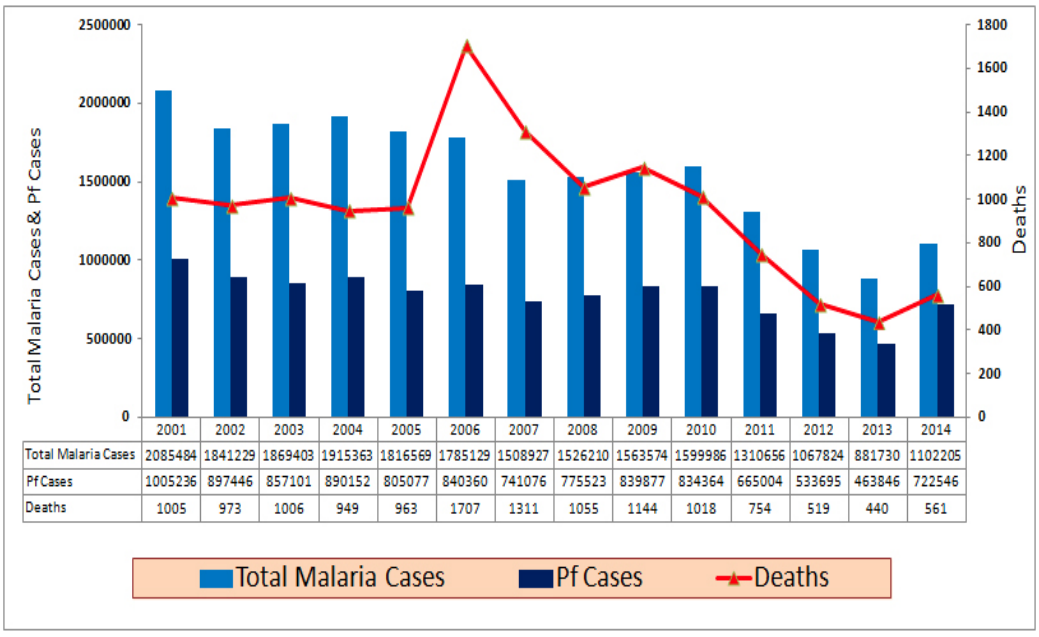


Figure 1.1. Global malaria transmission (http://www.worldwatch.org/system/files/EP153A.pdf).



	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Total Malaria Cases	2085484	1841229	1869403	1915363	1816569	1785129	1508927	1526210	1563574	1599986	1310656	1067824	881730	1102205
Pf Cases	1005236	897446	857101	890152	805077	840360	741076	775523	839877	834364	665004	533695	463846	722546
Deaths	1005	973	1006	949	963	1707	1311	1055	1144	1018	754	519	440	561

Figure 1.2. Malaria burden in India showing the overall endemicity of malaria in the country (Adapted from http://nvbdcp.gov.in/malaria3.html).

1.2 Malaria vectors in India

Of the 60 morphologically distinct species in India, 9 are incriminated as vectors. Six recognized as primary vectors of malaria are *Anopheles stephensi, Anopheles culicifacies s.l., Anopheles fluviatilis s.l., Anopheles minimus s.l., Anopheles dirus s.l,* and *Anopheles sundaicus s.l.* Table 1.1 provides information pertaining to sibling species recorded and the existing members that are present in India, along with the ecological distribution attached to each species. *An. culicifacies* is responsible for 60–65% of the malaria burden (Sharma, 2012; Dash *et al*., 2008), *An. fluviatilis* for 15-20% new cases (NVBDCP, 2015) and *An. stephensi* for 12% of the total malaria cases annually (Tikar *et al*., 2011). India's huge expansive geography and highly diverse climate supported by the diverse malaria epidemiology is a factor for the plethora of morphologically indistinguishable malaria vector species complexes comprising cryptic species varying in biological attributes (Dash *et al*., 2007; Singh *et al*., 2010).

Table 1.1. Principal malaria vectors, sibling species and eco-distribution in India. (Modified from Singh *et al*., 2009).

Primary vectors	No. sibling species recorded	No. sibling species in India	Members present in India	Ecological distribution
An. stephensi			3 variants	Urban/peri-urban
An. culicifacies s.l.	5	5	A, B, C, D & E	Rural/peri-urban
An. fluviatilis s.l	4	4	S, T, U and V	Foothills/forest/ canal irrigated areas
An. minimus s.l.	3	1	*An. minimus s.s.*	Northeast states
An. dirus s.l	7	2	*An. baimaii, An. elegans*	Northeast states
An. sundaicus s.l.	3	1	New cytotype D	Andaman-Nicobar

1.3 *Anopheles (Cellia) stephensi* Liston

Anopheles stephensi has been classified in Neocellia Series under Subgenus Cellia of genus Anopheles (Harbach, 2005). This sub-tropical species has its distribution extending from the Indian subcontinent (except Nepal and Sri Lanka) to the Middle East Afghanistan, Bahrain, Bangladesh, China, Egypt, India, Iran, Iraq, Oman, Pakistan, Saudi Arabia, and Thailand. Figure 1.3 shows the predicted distribution of the species in the Indian subcontinent. Three forms of *An. stephensi* have been

described, i.e., 'type form', '*var. mysoriensis'* and 'intermediate' based on egg ridges. (Subbarao, 1987). The rural *var. mysoriensis* is predominantly zoophilic preferring to rest outdoors in cattle sheds, barracks or poorly constructed houses, breeding in fresh water ponds, stream beds, seepage canals and wells (Subbarao, 1987).

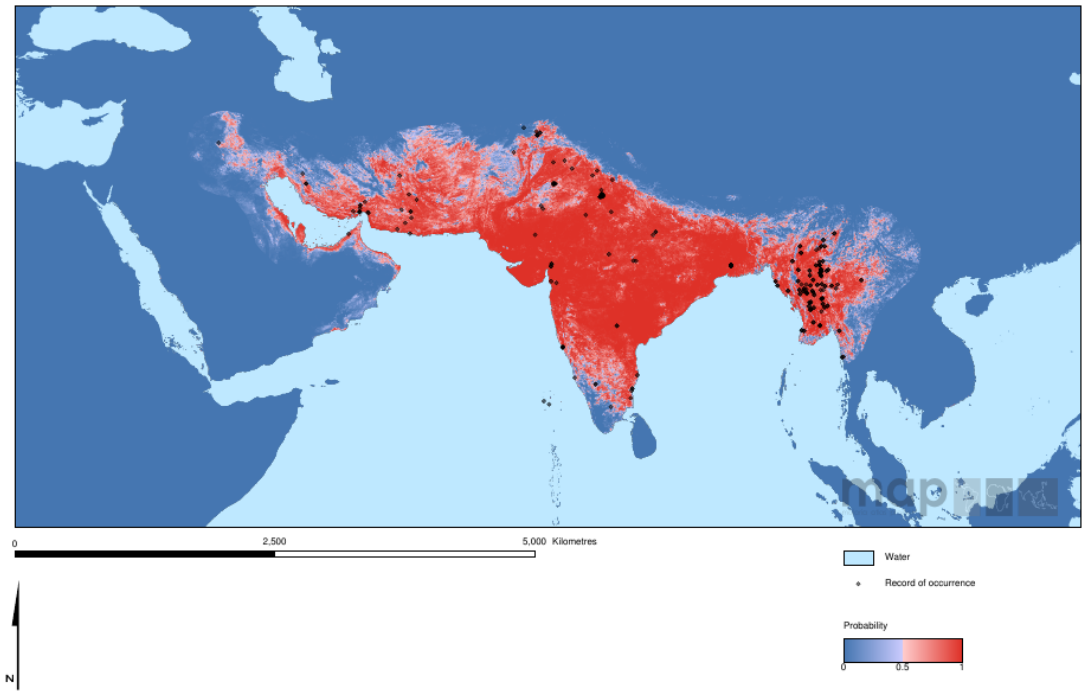


Figure 1.3 Predicted distribution of *Anopheles stephensi* covering the Indian sub- continent. Red indicates where the model predicts that the probability of finding the mosquito species is high and blue areas are where the model predicts that the probability of finding the species is low. Regional versions of this map also display species occurrence data (the black dots) as reported in the published literature- Sinka *et al*. (2011).The dominant Anopheles vectors of human malaria in the Asia Pacific region. These predictions were generated using the Boosted Regression Tree modelling methodology which also produced a ranked list of environmental variables assessed to be influential in predicting the presence of this species. (Adapted from Malaria Atlas map 2010).

1.4 *Anopheles stephensi* as a malaria vector

Anopheles stephensi is an important vector of malaria in the Middle East, Myanmar, China and India with the exception of Bangladesh (Dash, 2007) and more recently in the horn of Africa (Faulde *et al*., 2014). In India it is found throughout year with abundant numbers during the monsoon months of June through August, a period, which coincides with the transmission period (Sinka, 2011). Of the three forms of *An. stephensi, the* 'type form' is said to be responsible for malaria outbreaks in urban areas due to its association with construction projects and tropical aggregation of labor from

malaria endemic areas. Covell, in 1928 attributed its recorded high breeding as a cause of epidemics in metropolitan Mumbai. The rural occurrence of *An. stephensi* is also

Figure 1.4 An adult female *Anopheles stephensi* mosquito hand feeding at the in-house insectary at the National Institute of Malaria Research in Delhi (Photo credit: Dr. OP Singh).

considered as an important vector as seen in Iran (Subbarao, 1998) and in arid zones of Rajasthan, India (Dash, 2006). As an invasive species it makes entry into new towns and settlements, which are annexed to big cities. Lately, it has been recorded in the Northeast part of India (BN Nagpal, personal communication). The species is endophagic with its peak biting activity recorded between 22:00 to 24:00 hours although this varies seasonally in different localities (Dev, 2013). It is identified by pale markings on the wings, at least four dark areas are on the costa; with femora and tibia speckled, a close up of the lab strain with clear markings is shown in Figure 1.4.

Selection of this species for our molecular studies was motivated by reports of resistance in this vector to some of the insecticides, which are recommended for use in public health. Confirmed cases of DDT and malathion resistance in *An. stephensi* were recorded as early as in the 1980s in Delhi (Sharma *et al*., 1986), Goa (Thavaselvam *et al*., 1986) and from rural Haryana (Subbarao *et al*., 1994). Resistance to DDT with tolerance to malathion and permethrin was confirmed in Rajasthan (Bansal *et al*., 1996), and to malathion with tolerance to deltamethrin in Karnataka (Tiwari *et al*., 2010) with resistance to chlorpyriphos and to fenthion recorded in larvae collected from Rajasthan and Gujarat (Tikar *et al*., 2011). It is also indicated in a recent survey in India that resistance to synthetic pyrethroids is slowly developing in this vector, including *An. culicifacies* (Singh et al., 2014). Our previous study on the distribution of knock down resistance (*kdr*) mutations in India revealed a focal

distribution pattern exhibited by these mutations where the Delhi- national capital region, Haryana and Rajasthan populations harboured the 1014F/S mutation. Populations in the southern part of India which include Karnataka, Tamil -nadu, and Goa revealed the absence of *kdr* mutations (Dykes *et al*., 2016). Foreseeing the difficulties that will arise in malaria control programmes due to increasing resistance in this malaria vector, innovative vector control strategies are needed since the conventional methods such as IRS and LLINs though effective are operationally difficult and logistically demanding in urban settings. An understanding of the resistance mechanisms operating in *An.stephensi* to insecticides is of basic importance for designing better and improved vector control strategies.

1.5 Vector control

Vector control in malaria is an indispensable tool for eradicating the disease. Renewed efforts to control the disease from spreading worldwide has been led by the use of long-lasting insecticidal nets (LLINs), artemisinin-based combination therapy (ACT) and indoor residual spraying of insecticide (IRS), (WHO, 2008). From a global stand-point, vector control is achieved majorly through IRS employing DDT and pyrethroids, with the latter as the insecticide group of choice. In India, IRS is not employed in urban areas but recommended measures include (i) source reduction, (ii) minor engineering interventions (water storage designs) (iii) anti-larval methods, which include chemicals (temophos, fenthion) and biological larvicides, (iv) the application of larvivorous fish, eg. guppy fish *Poecilia reticulata* and mosquito-fish *Gambusia affinis*, (v) aerosol space spraying for control of adult vector populations, and (vi) legislative bylaws for preventing mosquito breeding (NVBDCP, 2013). In the face of rapid urbanization, unplanned growth, mushrooming urban slums and unsafe water storage practices, urban malaria became a growing problem. At present, urban malaria accounts for >10% of reported malaria cases in the country (Dev, 2013). Overall, malaria cases in the rural and urban areas are grossly underestimated due to scanty surveillance and unreliable laboratory services. DDT, pyrethroids and malathion for adult control, and temophos and fenthion as larvicides are presently used in India and form the principal insecticides commonly used in vector control programme. The public health sector under the government of India has recommended the use of pyrethroids as the principle insecticides where the registered insecticides for use since the year 2015 are given in Table 1.2.

Table 1.2. Principal insecticides registered for use in India in 2015 (Adapted from Ministry of agriculture list of registered Insecticides. Govt. of India.).

Insecticide	P/H	Type	a.i. (mg/m^2)	Formulation (gm)
Alphacyper-methrin 5 % wp	PH	Adult	25 (2 cycles application to repeat after 3 month) 40 (single cycle application)	Dilute 250 gm of Alphacypermethrin 5 % WP in 10 litre of water to cover500 sq m area
Azadirachtin 0.15% ec	PH	Larva		
Bacillus sphaericus 1593 m sero type h 59 5b	PH	An sp culex sp	112	1 ltr/10 ltr of water
Bacillus thuringiensis var. *Israelensis* 0.5%WP	PH		0.75mg/m2	
Bifentthrin	PH	Adult	25 (2 rounds of spraying 3 months apart)	125/500m2
Bti 12% AS (Vectobac)	PH			
Chlorpyriphos methyl 40% ec	PH	Adult		
Cyfluthrin 10% wp	P	Adult	25 (2 cycles Application to be Repeated after 3mths. 40 (single cycles application)	200 or 400
DDT 50% WP	PH	Adult	1-2gm/m	
Temophos 50% EC	PH	Larva	37.5-125	75-250 and waiting period of 200 days
Pirimiphos methyl 50%ec	PH	Larva	25ml/ha	
Deltamethrin 0.15% + Piperonyl .55%	P	Adult		
Deltamethrin 1.25% W/W or 1% W/V	PH	Adult	0.5 gm (thermal fogging/ultralow volume application)	50 ml
Deltamethrin 2.5% WP		Adult	625-1250 mg/50 sq.m	25-50g/50 sq.m
Deltamethrin impregnated bed net	P	Adult	55mg/m2 (for import only)	
Diflubenzuron 2% Gr	PH	Larva	1.25-3 kg/ha	
Fenitrothion 40% WP	PH	mos/ flies	400gm	1000ml
Fenthion 82.5% 2%	PH	Larva + Adult Larva	95 gm (upto 10cm depth surface)for larva 124 100 500	115ml 150 5 (10cm depth) 25 (.5 mtr depth)
Lambda cyhalothrin 10% WP	P		7.5-15 gm 500m3	75-150 gm
Malathion 25% WP	PH	Adult	2 per sq. m	8 per sq. m (rep after 6-8 weeks)
Pyriproxyfen 0.5% Gr	PH		10 (0.01ppm) clean water or20 (0.02pppm) Polluted water	2 Between 3 weeks each 4

***Abbreviations: P- Public health; H- Household

In India, vector control measures are IRS (using DDT, malathion and pyrethroids) and LLINs for rural settings and larvicide (temophos, BTI for Aedes), biological control and pyrethrum in case of an epidemic in urban settings. Insecticide resistance poses a challenge to vector control, in case of failure of malathion (which serves as an alternative to DDT) and pyrethroids (alternative for failure of DDT and malathion). Refutation and low acceptance of certain communities towards IRS and bed nets owing to ethical and personal reasons has added to the challenge.

DDT resistance surfaced as early as five years post introduction of DDT in the 1950s to combat malaria (Rajagopalan *et al.,* 1956). Presently, most malaria vectors are resistant to DDT though comparably susceptible to malathion, with low resistance to synthetic pyrethroids (Singh *et al*., 2003; Tikar *et al*., 2011; Singh *et al*., 2014). *Anopheles stephensi*, the vector accounting for 12% malaria cases in urban India is resistant to DDT and developing resistance to pyrethroids (Tikar *et al*., 2011). While rural malaria control programs exist in India, there are no confirmed report on rural malaria focused on *An. stephensi* as a contributing vector found in the rural dry arid desertic Rajasthan. However, modernization of villages in this region will most likely bring in malaria sooner. In urban areas, anti - larval measures, space spraying and fogging measures go hand in hand with *Aedes* control. Ignorant use of DDT in the rural-agricultural sector and wide scale application of pyrethroids in household and public health sectors in urban settings are bottlenecks to vector control programmes in effecting a delay or preventing the emergence and spread of resistance.

1.6 Mechanisms of insecticide resistance

Resurgence in vector-borne diseases reflects the molecular and physiological dynamics of an insect vector in their evolving resistance to insecticides, or drug failure in pathogens arising from resistance. Vector control as a global strategy component for monitoring vector borne diseases through insecticide applications is largely compromised due to resistance. The mechanisms of insecticide resistance in vectors are varied and call for a basic understanding of how they work. Insect vectors manifesting a resistance phenotype often exhibit multiple, complex mechanisms conferring resistance. The two major mechanisms of insecticide resistance are target site insensitivity and metabolic detoxification.

1.6.1 Target site insensitivity

A resistance mechanism arising from an altered target site(s) where the toxin-binding site becomes modified either through mutational changes from amino acids substitutions (Williamson *et al*.,1996) or through regulated activity of a gene post transcriptionally modified reducing the effects of an insecticide (Dong, 2007). Target site resistance genes encode essential proteins and limit mutational changes to very few, which would permit both resistance and viability (Wilson *et al*., 1998). An illustrative example is the voltage gated sodium channel (VGSC) gene, which harbours knockdown resistance mutations at the L1014 residue where substitution changes of bases from L-to-F/S results in a resistant insect phenotype for DDT and pyrethroids (Bloomquist, 1996; Martinez *et al*., 1998; Ranson *et al*., 2000). Other target site genes are acetylene-cholinesterase gene (*Ace-1*), and the gama – aminobutyric acid (GABA) receptor encoded by *Rdl* (resistance to dieldrin) gene. Point mutations in the *Ace-1* gene (Bass *et al*., 2014) and duplication and mutational changes including compensatory mutations in the GABA receptor sub unit (Zhang *et al*., 2016) are associated with insecticide resistance.

1.6.2 Metabolic Resistance

Metabolic resistance refers to the ability of resistant insects to detoxify or destroy the toxin faster than susceptible insects, thereby quickly ridding their bodies of the toxic molecules or sequestration of insecticides (Hemmingway, 2000). Such resistant strains generally constitutively overexpress metabolic enzymes or occasionally mutated forms with enhanced metabolic activity to break down a wide variety of insecticides may be due to certain enzymes having a broad spectrum of activity (IRAC). Monooxygenases, esterases and glutathione –*s*- transferase are classic examples of such enzymes transcribed by members belonging to large multigene families, which play a role in detoxification (Panini *et al*., 2016). Resistance in insects, exhibited from these enzymes can be due to gene amplification, which results in an increased amount of the enzyme. Gene amplification relating to pyrethroid resistance was reported in GST (Ranson and Hemmingway, 2005; Vontas *et al*., 2002) and in carboxylesterases (Field *et al*., 1988; Bass *et al*., 2014) leading to overproduction of the enzyme has had extensive study in *Myzus persicae.* Work on GSTe2, implicated in DDT resistance due to DDT dehydrochlorinase activity in major insect species and GSTe7, suggest that

these may also have a role to play in pyrethroid resistance (Lumjuan *et al*., 2011). Increased levels of P450s and enhanced monoxygenases activity is associated with carbamate, organophosphate and pyrethroid resistance in insects (Alptekin *et al*., 2016; Phillipou *et al*., 2010). Other metabolic mechanisms include the P-glycoprotein transporter, which takes advantage of the ATP hydrolysis based energy to translocate metabolites across the cell membrane (Hollenstein *et al*., 2007).

1.6.3 Other resistance mechanisms

Apart from target site insensitivity and metabolic mechanisms, others, which form the most recognizable ones, include behavioural resistance, where changes in behavior allow the insect to avoid insecticides, and physiological resistance. Physiological resistance includes cuticular resistance marked by decreased penetration or absorption through the insect wall due to changes in the insect cuticle (Lilly *et al*., 2016) from chemical interactions with the target system or localization of resistance implicated structural cuticular proteins such as CPLCG3 and CPLCG4 of *An. gambiae* (Vannini *et al*., 2014; Balabanidou *et al*., 2016), accelerated excretion- where the increased excretion of the insecticide is observed in the resistant strain (Zhao *et al*. 1994), increased sequestration or storage and slow turn-over of the insecticide evident in B esterase (Karunaratne *et al*., 1993) and carboxylesterase E4 enzyme overproduction (Hemmingway *et al*. 1998), which may be due to gene amplification. GST is also reported to provide protection against pyrethroid through sequestration (Kostaropoulos *et al*., 2000).

Further, multiple resistance mechanisms relating to DDT and pyrethroid resistance are reported in various insect species such as the African vector, *An. gambiae*, (Aikpon *et al*., 2014), *culex quinquefasciatus* (Li, 2013), *Aedes aegypti* (Kasai *et al*., 2014) and bed bug *Cimex lectularius* (Adelman *et al*., 2011). Co evolution of multiple mechanisms have also been shown, where P450 genes as orthologues of enzymes are involved in biosynthesis of cuticular hydrocarbon content (CHC), thereby playing a role in insecticide resistance via cuticular based resistance mechanism (Balabanidou *et al*., 2016). Studies in a DDT and pyrethroid resistant *An. gambiae* in Cameroon demonstrated that segregation of multiple resistance mechanisms resulted in heterogeneous resistance profiles (Nwane *et al*., 2013). Multiple resistance mechanisms heighten the insecticide resistance phenotype, which eventually could

widen the scale to multiple classes of insecticide posing an even greater challenge to current efforts in vector resistance management.

1.7 Insecticide susceptibility status of vectors in India

Consolidated data on vector susceptibility or resistance status of malaria vectors is lacking in India. The first report of DDT resistance in Indian *An. stephensi*, dates back to 1955 from a small town Erode, in Tamil Nadu (Rajagopalan *et al*., 1956), where it was used initially as a larvicide. In recent years *An. stephensi* has been reported to manifest resistance to multiple insecticides in different geographical locations (WHO, 1986). It is also found resistant to DDT, Hexachlorocyclohexane (HCH)/dieldrin (Kumari *et al*., 1998) and malathion in Haryana, Tamil Nadu, Karnataka, Gujarat (Singh *et al*., 2014), and to temephos in Kolkata (Chakravorty *et al*., 2000). Mittal reported the potential development of resistance in *An. stephensi* to microbial toxins found in *Bacillus sphaericus*, a bio-insecticide (Mittal *et al*., 2003). Scanty data, however, exists on the insecticide susceptibility status of *An. stephensi* to pyrethroids and malathion although these are the insecticide categories most tested. Knockdown resistance mutations were reported in a number of species including *An. culicifacies* (Singh *et. al*., 2010), *An. stephensi* (Singh *et al*., 2011), Aedes (Kushwah *et al*., 2015) and in *An. subpictus* (Singh *et al*., 2015). Metabolic resistance involving GST is confirmed in *An. culicifacies, An. anularis* (Gunasekaran *et al*., 2011) originating from breeding sites where *An. stephensi* is also present. *An. stephensi* has been reported to be tolerant to deltamethrin where studies showed that the tolerant strain depicted a higher activity of alpha-esterase and GST enzymes (Ganesh *et al*., 2003). Recent studies in *An. stephensi*, reported differential expression of glutathione s-transferase enzyme in different life stages.

Susceptibility status of malaria vectors in India has been reviewed based on reports published during the past decade covering the period between the year 2000 and 2013 (Singh *et al*., 2014).The susceptibility status of the six primary malaria vectors in India from data reviewed over the last decade is given in Table 1.3. This table constitutes reports where insecticides were tested against malaria vectors as per the World Health Organisation (WHO) recommendations, and the resistance status recorded.

Table 1.3. Insecticide susceptibility of malaria vectors in India from 2000-2013 (adapted from Singh *et al*., 2014).

Species	State	Susceptibility status*		
		Organochlorine (DDT)	Organophosphate (Malathion)	Pyrethroid (Deltamethrin)
An. culicifacies	Maharashtra	R	VR	VR
		R	VR	S
	Chhattisgarh	R	R	R/S
		R	VR/R	VR
	Madhya Pradesh	R	VR	VR
		R	R/S	R/S
		R	-	-
	Gujarat	R	-	R/S
	Jharkhand	R	S	S
		R	VR	S
	Rajasthan	R	R/S	S
	Uttar Pradesh	R	VR	S
		R	-	-
		R	S	S
	Odisha	R	R/S	VR/S
		R	S	-
		R	R	-
	Uttaranchal	-	VR/S	-
	Tamil Nadu	R	-	R
	Karnataka	-	VR	-
An. fluviatilis	Maharashtra	R	VR	VR
	Jharkhand	R	S	S
	Odisha	VR/S	VR/S	S
An. stephensi	Gujarat	R	R	VR/S
	Rajasthan	R	R/S	VR/S
	Karnataka	S	R	VR
	Kerala	R	S	S
	Madhya Pradesh	R	VR	-
An. minimus	Assam	VR	-	S
	Tripura	VR	-	S
	Odisha	VR	-	S
		R	-	S
An. annularis	Maharashtra	R	VR	VR
	Jharkhand	R	S	S
		R	VR	S

*As per WHO criterion based on mortality where if mortality is 98–100%: Susceptible (S); 81–97%: Verification required (VR) ;< 80%: Resistant (R).

1.8 Challenges in vector control management

Vector-borne diseases exist as the major public health concerns in resource-constrained settings and impoverished sections of the Indian society. Over the decades, vector control has emerged as an important tool, which helped minimize vector-borne disease burden worldwide. It relies to a great extent on synthetic insecticides as a mainstay in the war against vectors, with malaria almost completely eradicated in the 1950s through the use of the insecticide DDT. Unfortunately, reports on insecticide resistance impacting IRS effectiveness and malaria transmission is inconclusive and not fully understood, especially in endemic areas exhibiting diverse epidemiological

settings like Africa and India. This is partly due to the absence of reliable surveillance data and disease estimates and a complacent approach to the disease. Vector control has proven its effectiveness in containing the disease as evident in African nations when more than 663 million cases were averted since 2000 with a 40% fall during 2000-2015. Insecticide treated nets (ITNs) intervention emerged as the largest contributor averting 68% of the cases (Bhatt, 2015).

Pyrethroids, being the only approved class of insecticides recommended for use in ITNs, may prove disadvantageous in the long run, as the use of a single insecticide is likely to drive resistance. Reports on wide spread resistance to pyrethroids in *An. gambiae* (Knox *et al*., 2014; Ranson and Lissenden, 2016) and *An. funestus* (Mulamba *et al.,* 2014) signals the emergence of pyrethroid resistant mosquitoes, which very likely can lead to evolution of large scale resistance (Wondji, 2012).

In India, control of malaria is focused on the control of *An. culicifacies,* where 60—70% of the allotted budget is spent on the control of malaria in areas where it is the major vector species for malaria transmission (IRS NVBDCP, 2009). Vector control measures are IRS (using DDT, malathion and pyrethroids) and LLINs for rural settings and larvicide (temophos, BTI for *Aedes*), biological control and pyrethrum sprays in case of an epidemic in urban settings. Insecticide resistance poses a challenge to vector control, particularly with no alternative insecticide available as mentioned earlier in case of failure of malathion (which serves as an alternative to DDT) and pyrethroids (alternative for failure of DDT and malathion). Refutation and low acceptance of certain communities towards IRS and bed nets owing to ethical and personal reasons has added to the challenge. DDT resistance surfaced as early as five years post introduction of DDT in the 1950s to combat malaria (Rajagopalan, 1956). Presently, most malaria vectors are resistant to DDT though comparably susceptible to malathion, with low resistance reported to synthetic pyrethroids (Singh *et al*., 2003; Tikar *et al*., 2011; Singh *et al*., 2014). The issue of insecticide resistance in Indian vectors therefore calls for a thoughtful methodical approach.

Evolution of resistance in vectors is normally identified by elevated metabolic catalysis and reduced target site sensitivity (Li, 2007). Resistance at the molecular level is shown by the harbouring of resistant alleles L1014F/S in the molecular target-- the voltage gated sodium channel (VGSC) (Martinez *et al*., 1998; Ranson *et al*.,

2000). Positive selection and footprint selection (Lynd *et al*., 2010; Jones *et al*., 2012) and allele fixation (Mathias *et al*., 2011) signifying the steady increase of *kdr* alleles with the use of pyrethroids can lead to an insecticide driven selective sweep as evident in Kenya (Lynd *et al*., 2010; Wang, 2015). This emphasizes the importance of *kdr* in the evolution of pyrethroid resistance with metabolic detoxification acting alongside (Mitchell *et al.,* 2014). Important metabolic insecticide resistance mechanisms include, cytochrome P450 monoxygenases (P450) mediated detoxification, GSTs) and hydrolases or esterases (Liu, 2007). While *kdr* allelic variation is associated with target site insensitivity, variations in the DNA of genes coding for enzymes are also found play a significant role in enhanced metabolic mechanisms.

Genomic changes such as polymorphisms and copy number variations underlying detoxification mechanisms in disease vectors remains poorly investigated (Faucon *et al*., 2015). Such changes can lead to gene duplication and amplification, overexpression and coding sequence variation in genes coding for metabolic enzymes such as esterases (Hemingway, 2000), GSTs (Riveron *et al*., 2014) and P450s (Berge *et al*., 1998) and some of, which are transposon mediated (Chen *et al*., 2015). Molecular mechanisms which explain enhanced enzyme activities (Yu, 2014) include mutations in cis-acting promoter elements or in trans regulatory loci, coding sequence changes due to point mutations, chimeric genes (combination of portions of different genes giving rise to new genes) (Joussen *et al*., 2012), duplications (overexpression of a resistant gene) (Puinean *et al*., 2010) and gene amplification (Wondji *et al*., 2009; Schmidt *et al*., 2010). Insecticide detoxification genes, classic example being P450s, may share an evolutionary association with genes involved in allelochemical metabolism (Li *et al*., 2007). Genomic characterization and gene expression analysis along with metabolic enzymes characterization will help provide a better insight to the mechanisms operating behind resistance, which is crucial not only in the development of efficient control programmes but also in the designing of novel strategies and tools aimed at combatting the evolution and spread of insecticide resistance.

1.9 Aims of the study

Anopheles stephensi has been used as a model in various host–parasite interaction and cross-talk studies besides creation of a transgenic variant, inefficient to harbor malaria parasites. However, the identification and characterization of resistance mechanisms

in this vector, which is pivotal to insecticide resistance and curbing malaria transmission, is largely left to be explained. Point mutations, such as *kdr* affecting insecticide binding in the VGSC leading to decreased sensitivity of target site receptors were reported in the vector. However, examination for compensatory mutations, which may play a helpful role in reducing the fitness cost of the vector, or post transcriptional modifications reported to result in altered gating properties and channel kinetics of ion channels, has not been initiated. Further, enhanced metabolic detoxification from enzyme overexpression and gene amplification, or even mutations happening along protein-coding-gene sequence are frequently implicated in resistance and need investigation. Since the knowledge of insecticide resistance mechanisms involving molecular mechanisms as gene-gene interaction and gene regulation in this vector is not fully explored, this study aims to investigate the mechanisms of insecticide resistance that operate in DDT- resistant strains of *An. stephensi* with its main objectives enumerated below.

1. Characterization of glutathione S-transferase epsilon array in *An. stephensi*
    i. Study polymorphism in GST epsilon genes and examine its role in insecticide resistance
    ii. Study expression profile of GST epsilon genes in DDT resistant and susceptible individuals
    iii. Examine molecular mechanisms leading to enzyme overexpression
    iv. Functional characterization of major recombinant glutathione–*s*–transferase epsilon enzymes

2. Characterization of the DDT target site, the voltage gated sodium channel (VGSC) in *An. stephensi*
    i. Screen new knockdown resistance (*kdr*)-like mutations in gene.
    ii. Study post-transcriptional modifications-RNA editing and alternative splicing
    iii. Understand the role of the *kdr* gene in resistant strains and identify the *kdr* relationship in resistant and susceptible strains.

Chapter 2. MECHANISMS OF INSECTICIDE RESISTANCE IN LAB SELECTED DDT AND DELTAMETHRIN RESISTANT *AN. STEPHENSI*

Abstract

Anopheles stephensi, one of the primary malaria vectors in India, was colonized in the laboratory and selected independently for dichlorodiphenyltrichloroethane (DDT) and deltamethrin resistance through intermittent selection pressure against the insecticides for several generations resulting in LT_{50} and LT_{95} of 40 and 155 hours respectively against 4% DDT and LT_{50} and LT_{95} at 6 and 42 hours against 0.05% deltamethrin with a 23-fold deltamethrin resistance. The starting cohort of mosquitoes prior to selection pressure had both classic *kdr* mutations L1014F and L1014S, although in low frequencies (<10%). Laboratory colonization showed fixation of a single *kdr*-allele, 1014S, in selected DDT- and deltamethrin-resistant lines as well as in control mosquito lines, suggesting selection of 1014S-*kdr* allele in laboratory conditions. The other *kdr*-allele 1014F, showed poor survivability in the laboratory as our repeated attempts to colonize this line were unsuccessful. The selected DDT-resistant line showed high glutathione S-transferase and acetylcholinesterase activity while the deltamethrin-resistant line showed significant elevation of monoxygenases and non-specific esterase activity compared to susceptible mosquitoes. Synergistic assays with piperonyl butoxide (PBO) on selected deltamethrin-resistant strains, which was homozygous for the 1014S-*kdr* allele, lead to reversal of susceptibility, confirming the involvement of monooxygenase and esterases in deltamethrin resistance with a minor role of L1014S. Pre-exposure of the DDT-resistant line to PBO showed no reversal in resistance against DDT indicating the role of glutathione S transferase as a probable mechanism in DDT resistance. The laboratory selected deltamethrin-resistant *An. stephensi* showed moderate level of cross-resistance against permethrin (type I pyrethroid) and cyfluthrin (type II pyrethroid).

2.1 Introduction

DDT and synthetic pyrethroids (SP) are the main insecticides currently being used in malaria control programmes for Indoor Residual Spraying (IRS) (WHO, 2006). Synthetic pyrethroids are the only insecticide class used for insecticide treated

mosquito nets- the preferred method for vector control in India (NVBDCP, 2015). Though *Anopheles stephensi* has been reported to present a challenge for vector control due to the species developing resistance against DDT (Rajagopalan *et al.,* 1956; Davari *et al*., 2007), reports of pyrethroid resistance in this vector are lacking in field populations in India. An understanding of the mechanisms which play a role in insecticide resistance in this vector is of key importance for an effective resistance management programme. The focus of this study is to improve our knowledge of the mechanisms of resistance through genetic selection of highly resistant DDT and pyrethroid (deltamethrin) strains in the laboratory.

This chapter is centered on identifying the important mechanisms of insecticide resistance in *An. stephensi* vector to understand their influence on resistance phenotypes. While it is essential to identify the different causative factors of resistance exhibited in field populations, studies on field population of *An. stephensi* are hampered by the seasonality of the vector, a lack of a pyrethroid resistant strains and an unpredictable vector control program. Thus, colonized laboratory strains are useful as a proxy in a controlled environment to investigate the types of genetic events and timescale that can give rise to insecticide resistance when the species is subjected to insecticide selection. Here, DDT and pyrethroid (deltamethrin) resistant strains were raised to help predict some of the resistance factors that might arise in the field, for informed resistance monitoring.

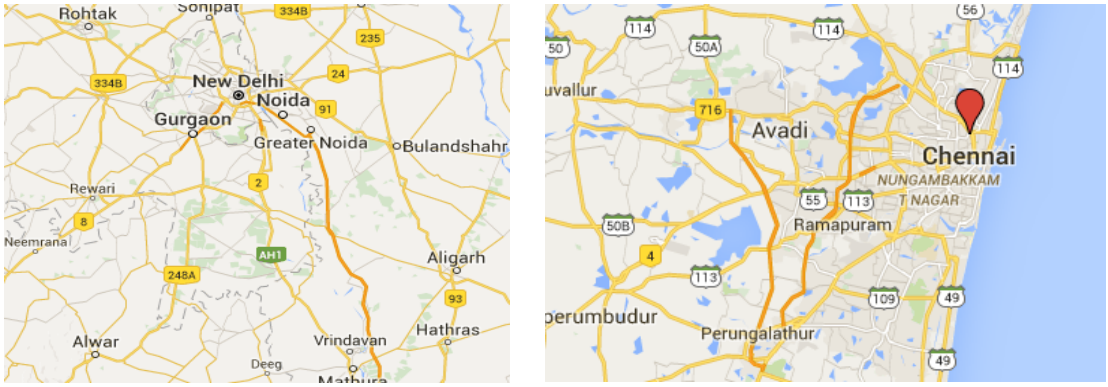
It is hoped that the *An. stephensi* resistant lines could provide an insight into the different resistance mechanisms involved, providing leads for candidate resistance genes. A series of basic WHO standardized experiments for detecting insecticide resistance mechanism were performed starting with the generation of L1014 mutant allele lines, and carrying out insecticide susceptibility tests including synergistic assays on homogeneous lines against DDT and deltamethrin and evaluating *kdr* association between them, as well as biochemical detection of elevated enzymes implicated in metabolic resistance. These experiments were performed with an aim to outline the basic mechanisms at work in the laboratory strains of *An. stephensi*.

2.2 Material and methods

2.2.1 Mosquito field collection and rearing

Anopheles stephensi were collected from villages in Umrain primary Health Centre of Alwar (27°26'-27°29'N and 76°31'-76°35'E) district of Rajasthan, National Capital Region (NCR) which also included Gurgaon (28°27′22″N and 77°01′44″E) city of Haryana and from Chennai (13°5′N, 80°16′E), capital city of Tamil Nadu (Figure 2.1).

Figure 2.1: Collection sites for initial collection of *An.stephensi.*



Adult female mosquitoes were caught from human dwellings and cattle sheds with the help of a mouth aspirator and a torch while immatures (larvae and pupae) were collected from natural breeding sites, mainly cemented tanks (overhead, fountains, underground water storage tanks, cisterns, flower pots and other temporary receptacles), and building construction sites using a dipper. Mosquitoes were brought to the laboratory and larvae were transferred to white enamel trays containing fresh water with fish food flakes and yeast powder, and were reared until emergence.

Rearing was done keeping insectary conditions at 27 ±1ºC temperature, 70-75ºC relative humidity and a 12:12 daylight photoperiod. Pupae formed were placed in a plastic cup containing water and kept inside a cloth cage measuring 30X30X30 cm allowing their emergence into adults. Upon emergence, the mosquitoes were given access to water soaked raisins and a wet cotton pad. Adult and emergent mosquitoes were morphologically identified using Christopher's' key (Christopher, 1933). Essentially, identification is by observations based on pale markings on the wings, tip of the hind tarsus which is not white, at least four dark areas are on the costa; pale

broad bands on the tarsus of front legs with femora and tibia speckled, two broad apical bands with one narrow and more basal band on female palpi and thorax with broad scales. For egg laying, mosquitoes were fed on a restrained rabbit placed inside a cage and feeding is allowed 1-2 hours for mosquitoes to feed to repletion. For oviposition, cups lined with filter paper and half-filled with water were placed in a cage 24 hours post blood meal to allow oviposition. Eggs were allowed to hatch and larvae were reared as described above. For obtaining isofemale progenies, blood-engorged mosquitoes were placed in individual plastic cups containing water and lined with filter paper. Eggs were processed for rearing as mentioned above and ovipositing females were preserved in isopropanol for molecular studies.

2.2.2 Insecticide susceptibility tests

Four-to-six day old F1 adult females were used for adult bioassay following WHO standard insecticide susceptibility test kit and insecticide papers having recommended diagnostic doses. Technical grade insecticides used in this study were DDT 4%, deltamethrin 0.05%, permethrin 0.75% and 0.15% cyfluthrin which were either procured from WHO distributor, Universiti Sains Malaysia or prepared in the laboratory at the Liverpool School of Tropical Medicine (LSTM) (DDT 4% and deltamethrin 0.05%). Whatman paper measuring 12 cm×15 cm impregnated with 2 ml mixture of acetone and a non-volatile carrier, olive oil (final concentration of on paper) for Organophosphate (OP) insecticides and DDT, and silicon oil (3.6mg/cm^2 final concentration) in case of pyrethroids was used. Impregnation was done by evenly pipetting the solution onto the filter paper which was then air dried in a fume hood and stored until use.

Up to twenty five female mosquitoes per replicate in three replicates were exposed to impregnated papers of diagnostic doses of DDT (4.0%), permethrin (0.75%), deltamethrin (0.05%) and 0.15% cyfluthrin for 1 hour, and then transferred to a recovery tube provided with 10% glucose soaked cotton pad. Each group has an appropriate control maintained using untreated paper. Mortality was recorded post-24 hours, following which, dead and alive mosquitoes were separated and preserved for experiments. To determine the susceptibility status of the vector, WHO criterion (2013) was considered where mortality range >98% -100% is considered susceptible, between 90 –98% mortality suggests the existence of resistance which needs further

investigation, and < 90% mortality confirming resistance. For determination of lethal time values, (LT_{50} and LT_{95}), exposure was made at different times starting from 0.125 to 8.00 hours in geometric series intervals. Knock down time of mosquitoes for insecticide was recorded. Data was analyzed by regressing log-dose and probit mortality using statistical software SPSS (IBM, USA).

2.2.3 *kdr* genotyping

Knockdown resistance genotyping of *An. stephensi* was carried out following PCR based assays described (Singh *et al.,* 2011). Two allele-specific PCR assays, PCR-F and PCR-L/S were employed to detect *kdr*-alleles. PCR-L/S discriminates 1014F from all other alleles (wild and 1014S) and PCR-F discriminates 1014S from L1014. PCR reaction comprised a mixture of 1X buffer, 1.5mM MgCl2, 200 µM of each dNTP, 0.375 units of AmpliTaq Gold taq polymerase and primers. Primers used for PCRF were 0.50 µmol of St-PheR (5'- GAT CGG AAA GTA AGT TAC TTA CGg CA -3'), 0.25 µmol of St-L/SR and 0.25 µmol of St-F ((5'- GAT TGT GTT CCG TGT GCT GT -3'), and primers for PCR-L/S were 0.50 µmol each of St-F, St-L/SR (5'- GCG GGC AGG GCG GCG GGG GCG GGG CCC GAT CGG AAA GTA AGT TAC TTA CGt CT -3'), St-LeuR (5'- GCG GGC AGG GCG GCG GGG GCG GGG CC C GAT CGG AAA GTA AGT TAC TTA CGA **g** TA -3') and St-SerR(5'- CGA TCG GAA AGT AAG TTA CTT ACG AtT G -3') (A tail of 26 bp was incorporated in primer St-L/SR and St-LeuR).The thermal cycling conditions for both PCRs were: one pre-denaturation cycle at 95°C for 5 min; followed by 35 cycles each at 95°C for 30 S, 55°C for 30 S and 72°C for 45 S, and a final extension step at 72°C for 7 min. Positive and negative controls were used for all PCR assays.

2.2.4 Selection of lines for *kdr*- genotypes and DDT-Deltamethrin resistant phenotypes

DDT and deltamethrin resistant line

A cohort of >400 mosquitoes collected from Alwar, which showed tolerance to deltamethrin (97% CM on one hour exposure against 0.05% DEL) and resistant to DDT (77% CM on one hour exposure to 4% DDT) were split into two cages. One of each were subjected to intermittent exposure to insecticide treatment (experimental) and the other without treatment (control) were maintained as continuous lines in the

study. For selection, 4-6 days old sugar-fed mosquitoes of a progeny were exposed to 4% DDT and 0.05% deltamethrin respectively at minimum exposure time under careful observation for alternate generations, which is then increased gradually. The time of exposure was initially 5 minutes which was increased up to 17 hours for selection against deltamethrin and 2 hours to 36 hours in the case of DDT.

kdr lines:

Single female progenies (~200 females) were obtained from parental lines (Alwar) and the final 2/3rd of abdominal segments were removed for genotyping of *kdr* alleles which also contains spermatheca containing DNA of male partner. Thus the excised portion had DNA from both parents. *kdr* genotyping was done following established protocols (Singh *et al.*, 2011). Adult progenies (arising from > 50 eggs laid per female mosquito) of all parents, which genotyped as LL, were pooled in a single cage and referred as the L1014-line. Selection of 1014F and 1014S lines were performed in two steps due to low allele frequency in the wild population. Firstly parents identified as L/F or L/S were pooled in different cages to enrich them for 1014-F or 1014-S allele. In the second step isofemale progenies of F1 were obtained and genotyped for *kdr*. Parents were identified as explained above, and progenies from parents bearing homozygous FF or SS were pooled in different cages.

2.2.5 Biochemical Assays

Biochemical assays were carried out on resistant lab strains, unselected and field collected Chennai susceptible strain. Twelve 4-6 day old unfed adult female mosquito in three replicates of DDT, deltamethrin resistant, unselected and susceptible strains were respectively assayed in order to compare levels of enzyme activity of mixed function oxidases (MFO), glutathione-S-transferases (GST), none specific esterases (alpha naphthyl acetate as a substrate), and acetylcholinesterase (AChE). Mosquitoes taken for the biochemical assays were never pre-exposed to any insecticides prior to carrying out biochemical experiments. Individual mosquitoes were homogenized in 200 µl of potassium phosphate (KPO_4) buffer (pH 6.5). The homogenate was divided and used for carrying out different enzyme assays, each in duplicates in a 96 well microtiter plate following the WHO Field and laboratory manual (WHO/CDS/CPC/MAL/98.6). The absorbance (Optical Density, OD) was measured spectrophotometrically using microplate plate reader, Spectrostar Nano^{TM} at

wavelengths as described for each enzyme. The mean absorbance was calculated for each replicate and the enzyme activity was recorded and compared. All biochemical reagents were procured from Sigma Aldrich.

2.2.6 Synergist bioassays

A total of eighty female mosquitoes (n=80), 20 per tube in 4 replicates of F1 adults of pyrethroid resistant *An. stephensi* and DDT resistant (n=60) in 3 replicates were pre exposed to WHO recommended 4% PBO (Universiti Sains Malaysia), an inhibitor of oxidases and esterases for one hour. The mosquitoes where then exposed to DDT (4%) and deltamethrin (0.05%) respectively for another 1 hour keeping a control group (DEM-/PBO-) using control paper. Post exposure, mosquitoes were transferred to holding tubes and given access to 10% glucose soaked cotton pad and kept for recovery for 24 hours. Mortality post 24 hours was assessed and compared to the results obtained from replicates without PBO treatment. This was carried out in order to understand the role monooxygenases or esterases might play on the DEL and DDT resistant colonies.

2.2.7 *kdr* fitness

Two hundred pupae (100 each males and females) each from two k*dr* lines L1014 and 1014S were pooled in a cage and placed in an insect cage for emergence and allowed to inbreed for seven generations. Post 7 generations, a cyclic colony was maintained using the experimented batch. Rearing followed as detailed earlier. We failed to colonize 1014F line and no pooling was carried out. Approximately 100 mosquitoes were sampled at F1, F3, F7 generation and post one year of cyclic colonization were genotyped for *kdr* allele.

2.2.8 *kdr* allelic association

A total of 61 *An. stephensi* were exposed to 0.05% deltamethrin impregnated paper using WHO's standard susceptibility tests kit for a period of one hour and were subsequently transferred to holding tube for recovery for 24 hours with access to 10% glucose soaked cotton. A maximum of 25 mosquitoes were exposed in a single tube. Dead and alive mosquitoes were genotyped for classic *kdr* alleles following Singh *et al.* (2011).

2.3 Results

2.3.1 Insecticide Susceptibility tests

Standard WHO susceptibility tests for DDT, deltamethrin and permethrin were performed on *An. stephensi* during the initial stages prior to selection experiments (Table 2.1). Post selection, susceptibility tests for the three insecticides but including cyfluthrin, an insecticide belonging to the pyrethroid class type II for the DEL selected strain showed significant increase in resistance. The DDT selected strain recorded 78% mortality against DDT on one hour exposure prior to selection. Post selection experiments, it was observed that no mortality was observed following standard susceptibility tests protocol recording an LT_{50} at 40 hours exposure. Further, resistance in the DEL selected strain can also be explained by a complete absence of mortality on one hour of exposure to the 4% DDT and 0.05% deltamethrin and a 65% increase in resistance to the permethrin. Although cyfluthrin susceptibility was not examined prior to selection, the results show that the DEL strain manifested resistance against this insecticide.

Table 2.1.Results on WHO susceptibility tests conducted at 1 hour exposure from initial field collection pre and post selection in the laboratory.

Insecticide/dose	% corrected mortality*	LT_{50} in hrs**	LT_{95} in hrs**
A. Pre-selection			
DDT 4%	77.5± 6.60	-	-
DEL 0.05%	97.5 ± 2.47	0.25 (0.18-0.33)	0.92 (0.57-1.50)
PER	100	-	-
B. Post-selection DEL line			
DDT 4%	0	14.40 (11.45-18.09)	68.52 (39.59-118.58)
DEL 0.05%	0	5.66 (4.28-7.49)	41.48 (18.96-90.74)
PER	35.0 ±7.86	1.09 (0.62-1.92)	18.12 (3.51-93.52)
CYF	30.0 ±7.65	1.48 (1.00-2.18)	17.68 (4.29-72.84)
C. Post-selection DDT line			
DDT 4%	0	39.67 (35.41-44.43)	154.82(114.46-209.4)

*On one hour exposure followed by 24 hr recovery; ** Figures in parenthesis indicate fiducial limits at 95% confidence interval.

It may be noted that the two strains-DDT and DEL were from the same origin which is the field collection originating from Alwar and NCR where reports of DDT resistance for this vector in Rajasthan is established with an adult mortality percentage ranging between 60-70% against 4% DDT and 93-100% in 2007 (Tikar *et al.,*2011). The fact that field populations examined in this study already show some level of DDT

resistance (77% CM) made it difficult to obtain a true baseline regression of time series data for the DDT resistant strain which is colonized in the laboratory. Furthermore, it was observed that exposure to DDT at the WHO recommended 4% dose for 1 hour had no effect on the colonized strain and the strain showed sustained resistance (~95% CM) even after exposure to DDT for 48 hours. This explains the high degree of DDT resistance in the DDT resistant laboratory strain with resistance also seen in the DEL resistant strain. While scanty reports of pyrethroid resistance exist in the field, the deltamethrin-resistant strain in our study, showed an increase in the resistance pattern where resistance observed in a few individuals is at 17 hours maximum exposure.

2.3.2 Synergistic bioassays

Treatment with the PBO synergist showed efficacy in enhancing the toxic effects of the insecticides against the DEL strain laboratory colonies. Synergist PBO against mixed function oxidases (MFO) and esterases in the DEL strain indicated that these enzymes may play a role in conferring deltamethrin resistance where reversal of susceptibility is recorded at 83% (Figure 2.2). DDT resistant laboratory *An. stephensi* showed no significant reversal of susceptibility when pre-exposed to PBO as a synergist with corrected mortality recorded at an insignificant 3% which indicates MFO as an unlikely mechanism to operate in the strain (Figure 2.3). In the case of P450, PBO is described capable of inhibiting esterases (Gunning *et al*., 1998; Herron *et al*., 2014; Young *et al*., 2004). In the DEL resistant *An. stephensi*, the high degree of mortality i.e., the reversal in susceptibility for the pyrethroid observed in PBO pre exposed replicates compared to PBO untreated with mortality at 7.5% strongly suggests a role for monoxygenases and maybe esterases in conferring protection against pyrethroids in the deltamethrin resistant laboratory strain.

2.3.3 Biochemical assays

Four- fold elevation of GST activity (1.8 µmol/min/ml) in DDT resistant strains compared to the susceptible strain (0.4µmol/min/ml), strongly indicates a possible GST based mechanism of resistance in DDT resistant lines (Figure 2.4). In the pyrethroid resistant non blood-fed *An. stephensi* laboratory strain that is the DEL strain, significant elevated levels of monoxygenases ($p<0.0001$) was observed. DEL resistant strain showed a three-fold elevation of monoxygenases level when compared to the susceptible controlled strain maintained under equivalent conditions, thus

suggesting metabolic resistance to be associated with P450 activity. No significant increased levels in monoxygenases or esterases were detected in DDT resistant or unselected strains of *An. stephensi* examined.

Multifunctional oxidase activity is a measure of the total haeme content of the mosquito. Since P450s are not the sole source of haeme, this assay is only suggestive of changes in P450 content. Synergist assay using PBO represented earlier (Figure 2.2) further confirmed their role restoring susceptibility to the pyrethroid by 83% corrected mortality. Acetylcholinesterase assay for the two resistant strains and the unselected strain showed significant difference on propoxur inhibition rates ($p<0.0001$) when tested against the susceptible as represented in the graph (Figure 2.4), which represents the percentage residual activity in propoxur inhibited fraction. Esterase activity measured by using naphthyl acetate end point assays show a high significant degree of the enzyme elevation in the resistant deltamethrin pyrethroid stain compared to the susceptible ($p<0.001$). However, inhibition tests were not conducted to confirm the role of esterases in this strain although PBO synergists have also been used (Khot *et al.,* 2008) against esterases to confirm their presence.

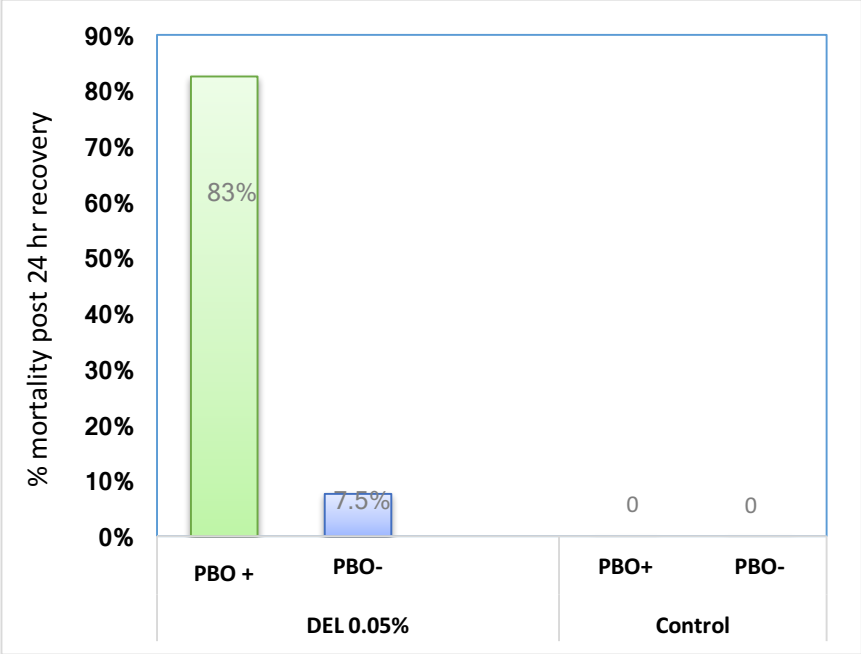


Figure 2.2. Synergistic effect of PBO on the toxicity of Deltamethrin in deltamethrin-selected *An. stephensi* n=80 post one hour exposure [with (PBO+) and without (PBO-) pre-exposure with 4% PBO for one hour].

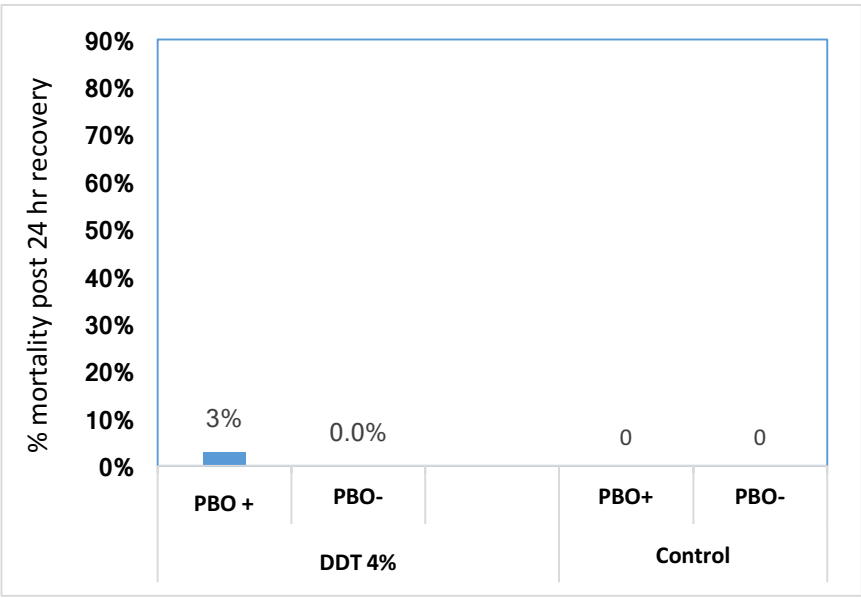


Figure 2.3. Synergistic effect of PBO in DDT-selected *An. stephensi* n=60 post one hour exposure to the insecticide [with (PBO+) and without (PBO-) pre-exposure with 4% PBO for one hour].

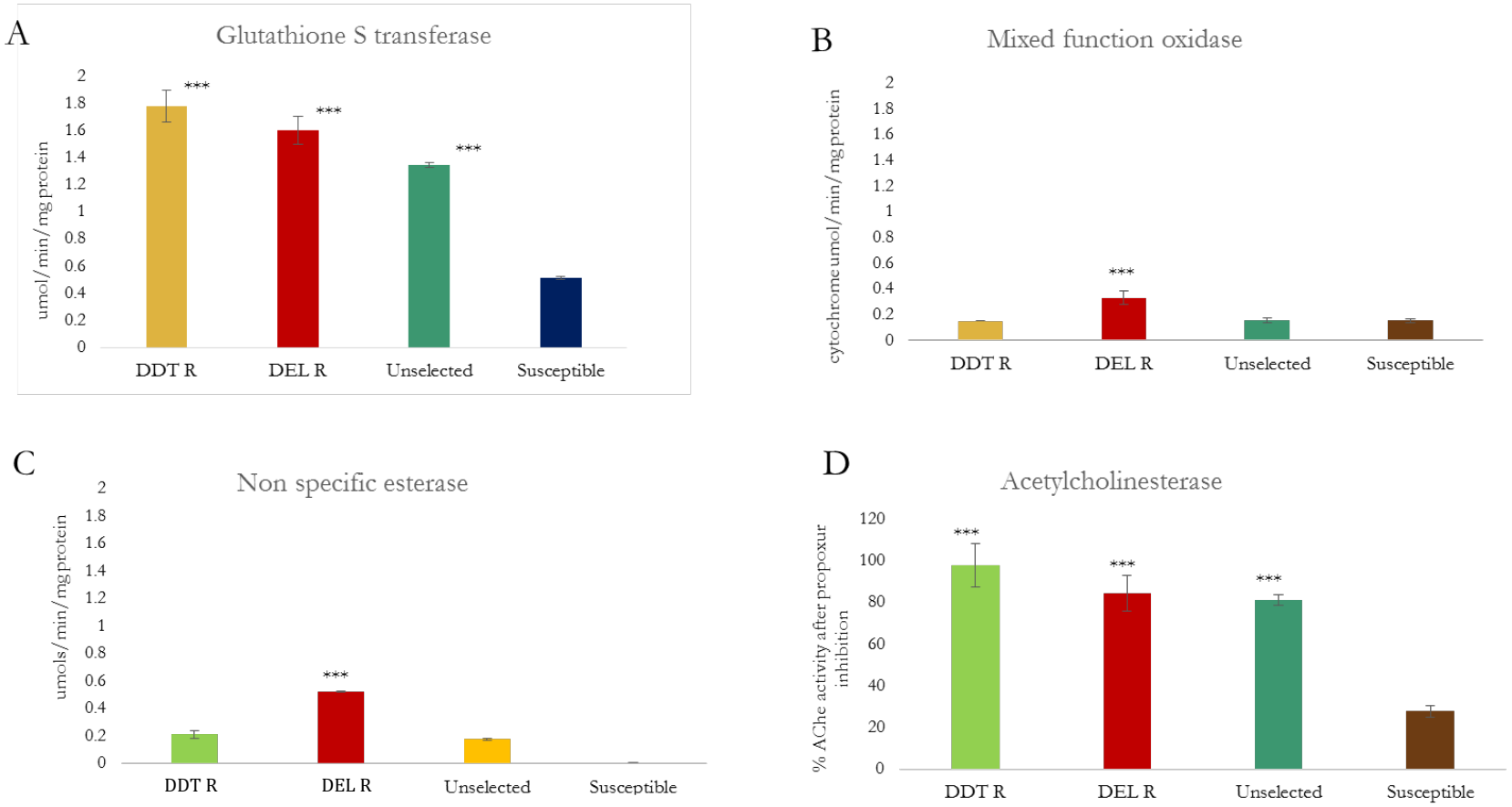


Figure 2.4. Chart showing GST enzyme activity (A), MFO activity (B) non-specific esterases activity (C) and % residual activity of AChE (D) in DDT resistant , DEL resistant , unselected and the susceptible *An. stephensi* laboratory strains Error bar depict standard error of mean and asterisks show high level of significance (p value ***=<0.0001; t-test with susceptible) n=24.

2.3.4 Fitness of *kdr* alleles in laboratory conditions

While it was observed that the frequency of L1014 and 1014S were almost equal in F1 with 1014S (48%) and L1014 (52%), it was seen that the *kdr* 1014S allele increased from 0.48 in F1 to 0.7 through F6 generation even in the absence of insecticide selection pressure (Figure 2.5). The resulting F6 was isolated and made to undergo inbreeding for several generations (homogeneous) and sampling was then performed which showed that *kdr* mutation L1014S completely replaced wild type (L1014) suggesting the high survivability of 1014S in laboratory condition compared to wild type. The analysis of Hardy-Weinberg equilibrium of two *kdr* genotypes L1014 and 1014S is shown in Table 2.2 where a departure from Hardy-Weinberg equilibrium (HWE) due to deficiency of heterozygotes L1014S in subsequent generations is observed. Further, a cyclic colony (colony in which we keep on adding *An. stephensi* progenies to the stock cage for the purpose of maintenance of a general pool), after one year of selection revealed a homozygous 1014S in all individuals genotyped. Noncompliance of HWE equilibrium further confirms ongoing selection process. Similar experiments on fitness of L1014F mutation could not be initiated because we failed to colonize L1014F in the laboratory. This indicates the high fitness cost of L1014F and the inability to adapt in laboratory conditions. It was observed that 1014S is present in *An. stephensi* laboratory DDT-resistant and deltamethrin-resistant strains, including the laboratory colonies which are free of selection pressure. Pooled proportions of L1014, and L1014S in equal numbers allowing for random mating for 7 generations led us to observe a high frequency of the 1014S allele, which indicates that the 1014S has a low fitness cost and is positively selected under laboratory conditions. Recent studies on the distribution of *kdr* alleles in India showed preponderance of this allele in Northern India in association with L1014F (Singh *et al.*, 2011; Dykes *et al.*, 2015)

Table 2.2. Temporal dynamics and test of Hardy-Weinberg equilibrium for *kdr* alleles in laboratory *An. stephensi* colony.

Generations		Genotypes			*p*-value	Allelic frequency	
		LL	LS	SS		L	S
F1	obs	32	37	29	<0.02	0.52	0.48
	exp	26.02	48.95	23.02			
F3	obs	20	41	47	<0.05	0.38	0.63
	exp	15.19	50.63	42.19			
F7	obs	10	25	66	<0.01	0.22	0.78
	exp	5.01	34.98	61.01			
Cyclic colony (after one year)	obs	0	0	30		0	1.00

Abbreviations: obs=observed, exp=expected

2.3.5 *Kdr* Genotyping and its association with insecticide resistance

Bio-assayed samples (dead and alive) post 24 hour recovery were genotyped for *kdr* mutations which revealed the presence of 1014S alleles in dead and alive mosquitoes in the DEL strain. It was also observed that DDT resistant strains of our cyclic colony is homozygous for 1014S allele. The role of classic *kdr* mutations in conferring resistance in the laboratory colonized strains is not significant, fisher's $p>0.05$ (Table 2.3) and needs deeper investigation before any conclusion is arrived. A recent study in Afghanistan reported a significant fraction of deltamethrin-resistant mosquitoes to be homozygous for the 1014L wild type allele with the indication that other mechanisms must be involved for the observed pyrethroid resistance in the region (Ahmad *et al.,* 2016).Our biochemical findings suggest the bigger role that metabolic resistance may have in laboratory resistance and perhaps in the field as well. *Kdr* allele 1014S show no protection in DEL strains but their role in DDT resistance cannot be ruled out completely.

Table 2.3. Allelic association of *kdr* alleles with DEL resistance in *An. stephensi* (Mosquitoes were exposed to 0.05% DEL for 60 mins) n-=61.

	***kdr*-genotypes**							**Test of allelic association**			
								Fisher's exact test		Odds ratio (fiducial limits at 95% CI)	
	LL	LF	LS	FF	SS	FS	total	L vs F	L vs S	L vs F	L vs S
Alive	8	1	10	0	0	0	**19**	0.2	0.74	0.36 (0.07-1.68)	1.15 (0.61-2.15)
Dead	20	3	16	0	3	0	**42**				

2.4 Discussion

Laboratory strains of *An. stephensi* in this study have undergone controlled selection with DDT and deltamethrin in order to examine the evolution of insecticide resistance mechanisms. Such strains are a useful tool for predicting the likely mechanisms of resistance in field populations under similar selection pressure. The widespread scale of insecticide resistance makes it necessary to review the mechanisms of insecticide resistance at the basic level for an effective integrated vector management (IVM). A sound knowledge of the molecular and metabolic basis of DDT and pyrethroid resistance is fundamental to developing new strategies for combating resistance.

Metabolic resistance mechanism is evident from biochemical and synergist assays in our laboratory raised DDT resistant and deltamethrin resistant *An. stephensi* strains. Glutathione *S*-transferase enzyme is significantly elevated in DDT-resistant strain where almost fourfold levels is observed compared to the susceptible strain. Significant elevation of GST in DEL-resistant and the unselected *An.stephensi* strains is also recorded in the study. Three fold elevated levels of monoxygenases and high esterase levels are a strong indication that these enzymes may play a role in the detoxification of deltamethrin in DEL resistant strains. This was supported by PBO synergist assays where susceptibility to deltamethrin was found restored in the resistant *An. stephensi* DEL strains resulting in high mortality greater than 80%.

Both esterases (Latif *et al.*, 2010; Young *et al.*, 2004) and P450s are known to be associated with pyrethroid resistance (Aizoun *et al.*, 2013; David *et al.*, 2013; Liu *et*

al., 2015).Thus, considering the high degree of resistance in DEL resistant strain there may be a combination action of MFO and esterase working together to produce a higher degree of resistance in the pyrethroid resistant strain. Acetylcholinesterase showed no significant difference between the laboratory strains although inhibition rates by propoxur were significantly different ($p<0.001$) for the susceptible Chennai. The fourfold difference in acetylcholinesterase between the laboratory strains and the susceptible in our case could be due to differences in strains and can very well be explained by adult resistance to malathion, or, larvae resistance to temophos both which are in use. Carbamates however are not applied in vector control in India (NVBDCP, 2010) While larval susceptibility study was not carried out, temophos resistance at early stage could not be ruled out. Altered acetylcholinesterase is known to be associated with organophosphate and carbamate resistance (Cuamba *et al.*, 2010). Cross resistance to cyfluthrin and permethrin is also observed in DEL-resistant strain where adult mortality 30% and 35% was recorded from susceptibility bioassays. Such cross resistance has been observed in this species but with OP compounds in a study where malathion-selection caused high cross resistance to fenitrothion while, fenitrothion selection produced high cross-resistance to malathion in a study in *An. stephensi* (Chitra and Pillai, 1984).

Attempts to underline the importance of selection pressure with regard to insecticide resistance levels only suggest that such resistance levels are not clearly quantifiable. Considering numerous factors, the highly resistant DDT and DEL strains selected in the laboratory show the highest resistance levels. Knockdown resistance alleles in DDT and DEL resistant strains of *An. stephensi* were investigated but whose role, in particular 1014S could not be firmly established. The homozygous 1014S allele with the highest survivability did not seem to have protect against deltamethrin resistance, quite evident from reversal of deltamethrin susceptibility in the DEL strain homozygous for the allele on treatment with PBO. Whether the fixation of 1014S in laboratory colonies is due to insecticide pressure or due to some other factor which aided in adapting population under altered (laboratory) condition, could not be established due to development of resistance in control mosquitoes which may be due to the presence of low level of residual insecticide in existing laboratory conditions.

It can be emphasized that the overall occurrence of *kdr* alleles and their frequency of distribution in India are still not at par compared to African countries where fixation of such alleles is no longer hypothetical but a fact reported in resistant populations. It also can be asserted that while *kdr* is not completely ruled out in terms of its role in conferring resistance in our study, a combinational action of knockdown resistance as well as metabolic resistance is probable primarily since *kdr* is considered a recessive trait (Martinez-Torres *et al.*, 1998; Ranson *et al.,* 2000). The presence of *kdr* 1014S in laboratory resistant strains initially suggests its probable involvement which however is challenged by the reversibility of resistance in such individuals on pre exposure to synergists. This became a disadvantage to our interpretation of *kdr* association with the phenotype and strongly suggests metabolic resistance as the major mechanisms operating in the selected colonies. In addition, fitness cost can act to prevent the fixation of new alleles which may play a defining role within a population. Further to it, while field populations show a relatively small frequency of *kdr* alleles in contrast to laboratory colonies, question on whether type form or mysoriensis harbours both mutations is left unexplained since exhaustive field collection is lacking although random field collections carried out in urban and rural settings showed the occurrence of mutations in both variants. While, monooxygenases strongly indicated its role in the pyrethroid resistance in DEL strain, esterases and GST seem to have a role as well. Supported by recent reports on the expression of multiple gene families as a factor in the escalation of pyrethroid resistance in *Anopheles coluzzi* in West Africa (Toe *et al.,* 2015), we may as well conclude that such event cannot be ignored in our pyrethroid resistant laboratory strain. The chapter outlines the defined role of metabolic GST in DDT resistant and possibly in DEL resistant *An.stephensi* strains.

Chapter 3. EVIDENCE OF TANDEM CO-DUPLICATION OF EPSILON-2 AND -4 IN GLUTATHIONE-*S*- TRANSFERASE GENE IN *ANOPHELES STEPHENSI*

Abstract

The glutathione *S* transferases (GSTs) are a multigene family of detoxifying enzymes against potentially genotoxic chemicals. In insects, GSTe2 is known to metabolize DDT in the presence of glutathione where resistance against DDT in a wide array of insects is ascribed to overexpression of this gene. Here, we show evidence of tandem gene duplication of GSTe2 and GSTe4 in the GST-epsilon array of a laboratory maintained DDT-resistant *An. stephensi* colony. The evidences include (i) unequal copy number of two AsGSTe2 variants in individuals, evident from DNA sequence chromatogram and variant-specific qPCR, (ii) presence of more than two haplotypes in individuals (iii) complete heterozygosity for variant alleles on two loci - one in the coding region of AsGSTe2 and another in the intergenic region (previously described e2 pseudogene) suggesting that variants are located on different loci in duplicated gene, and (iv) genomic copy number variations in individuals as detected by qPCR. We were able to identify duplication breakpoint, which revealed tandem duplication of a segment of the GST epsilon array in the DDT-resistant *An. stephensi* mosquitoes. The duplicated region comprises part of AsGSTe1, AsGSTe2, e2-pseudogene, AsGSTe4 and part of AsGSTe5 separated by a 2.7 kb DNA insert of unknown origin. On an average we found six copies of duplicated genes in DDT-resistant line evident from qPCR performed on AsGSTe2 where very low copy number AsGSTe2 was recorded in DDT-susceptible strain, indicating the possible role of gene duplication in DDT resistance. This chapter adds to our current understanding on the role genetic and molecular mechanisms have in insecticide resistance.

3.1 Introduction

The role of GSTs in insecticide resistance is not particularly clear. Qualitative and quantitative changes in the enzyme activity are associated with resistance to DDT, organophosphate (OP) and Pyrethroids (PY) (Ahmad and Forgash, 1976; Plapp, 1976; Li *et al.,* 2007). The role of GSTs in insecticide metabolism is thought to occur through

two routes- first through binding and sequestration of the toxin and second, by protection through by-products of toxicity from oxidative stress, as evident for pyrethroids (Vontas *et al.,* 2001, 2002). Riveron described a mutation L119F in the resistance incriminated GST epsilon 2 (GSTe2) as a driving factor for elevated enzyme activity in the *An. funestus* resistant strain in Benin (Riveron *et al.,* 2014). The arrangement of the GST epsilon cluster suggests evolutionary duplication of gene segments giving rise to paralogs. Tandem and segmental duplications were most likely the mechanisms playing behind paralog generation with a potential for genome size increase and for , which the duplicated genes with emerging functions then undergo positive selection for conservation (Cannon *et al,*. 2004). Although diversification in GST epsilon gene is derived from gene duplication resulting in an array of GST epsilon genes, duplication of a specific epsilon gene is unknown.

Gene duplication is a mechanism of genomic adaptation to stress and has been argued to have a contribution in reducing the risk of extinction via functional redundancy, mutational robustness, increased rates of evolution and adaption (Crow *et al*., 2006). The duplication event is seen in extensive diversification, which is exemplified in insect metabolic detoxification genes such as P450s (Wondji *et al.,* 2009; Emerson *et al.,* 2008; Li *et al.,* 2007) and esterases (Field *et al.,* 1998) involved in insecticide detoxification. These events are also reported in insecticide target genes such as the acetylcholinesterase receptor (*Ace-1*) (Constant *et al.,* 2014) and in the *Rdl* gene (Anthony *et al.,* 1998) functioning in nerve impulse transmission. The impact of *ace* gene copy number variations on resistance with duplication is described far more likely to occur in multiple resistant alleles than the susceptible ones with complexities in *An. gambiae (*Djogbenou *et al.,* 2015) and it is also described to play a role in providing new genetic material for mutation, drift and selection to act upon paving a way for evolutionary opportunities (Zhang *et al.,* 2003). It is of interest to understand if over production of detoxifying genes as a major mechanism of insecticide resistance is achieved from overexpression of a single copy of a gene or a mechanism of gene duplication. In this chapter, our aim is to bridge the gap and widen our fundamental knowledge of genetic events in insecticide resistance. We therefore investigated genomic events such as duplication and copy number variation in the GST epsilon gene to understand its precise role.

3.2 Material and methods

3.2.1 Mosquito samples

A highly DDT-resistant line of *An. stephensi* with an initial LT_{50} =14 hours was maintained in the laboratory and selected by exposing the mosquitoes to DDT in intermittent generations. This line originated from Alwar district, Rajasthan initially collected in 2012. DDT-susceptible mosquitoes were collected from Chennai in 2015 and were reared as a separate line, which served as a control.

3.2.2 Molecular characterisation of GST arrays

For amplification, cloning and sequencing of *An. stephensi* GSTe2 and GSTe4 genes, specific primers were designed for the full coding region of the epsilon gene with the help of GST epsilon reference genes downloaded from the *An. stephensi* genome database available from VectorBase. Initial amplification of the epsilon genes AsGSTe2 and AsGSTe4 was carried out by using flanking primers designed for *An. stephensi*. GSTe2F1 (5'-ACGAGCGCAAGTGAAATCAT-3') and GSTe2R1 (5'-ACGTTTGTGCTTCTTTATTTAA-3') for AsGSTe2 amplification and GSTe4F (5'-CTTGCAAGCGAGTGGAACT -3'), GSTe4R (5'CTTTGGGCTTTGAGCGTAGT-3') for AsGSTe4 gene amplification respectively. Internal Primers GSTe2F2 CTCCAACGACCACAATCATG and GSTe2R2 TGCTTCAAGTTACGTTTGTGC were designed for nested approach. GSTe2 and GSTe4 were amplified separately using 2ul cDNA in a 25ul reaction using Phusion High Fidelity DNA Polymerase (NEB, Ipswich, Massachusetts, USA) containing 1X buffer, 1.25 uM of each primer and 2U taq. The PCR conditions were an initial denaturation of 98ºC for 20 secs and a 30 cycle denaturation at 98ºC for 10 secs, annealing at 60ºC for 15 secs and extension at 68º C for 5 mins in ABI 9700 thermal cycler. Amplified product was cloned using CloneJET PCR cloning kit (Fermentas, Thermo Fisher) and sequenced at Macrogen Inc, South Korea.

3.2.3 Characterization of (ψAsGSTE2P) pseudogene

For amplification of e2-pseudogene intervening GSTe2 and GSTe4, PCR was performed on gDNA of DDT-resistant line using primers E2F (GCCGACTTTAGCTGCATCTC, designed from GSTe2) and PSR (CGA TCA GAT TGA TGG GCA CG-5, designed from GSTe4). The PCR was carried out with Phusion

High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs) using 0.1 uM of each primer. The PCR conditions were an initial denaturation at 94ºC for 3 min followed by 35 cycles each with denaturation step at 95ºC for 30 C and annealing/extension step at 68ºC for 2 min, followed by final extension at 68ºC for 10 min. Since direct sequencing was ambiguous due to the presence of several indels in GSTe2 and e2-pseudogene, the product was cloned and sequenced. Prior to cloning, the amplified product was purified using Qiagen PCR Purification kit (Qiagen Inc) and A- tailed by incubating the PCR product at 72ºC for 10 min in a reaction mixture containing 200uM of dATP, 1.5mM MgCl2, 0.625 unit of taq polymerase and 1X buffer. The PCR product was finally cloned in pGEM-T Easy Vector system (Promega Corporation) following the manufacturers protocol.

3.2.4 Haplotype identification

For the identification of the 3 suspected haplotypes identified after sequencing of a region encompassing the complete e2-pseudogene and flanking regions having partial GSTe2 and GSTe4, a PCR strategy was developed, , which was based on indels present at two loci, one in GSTe2 region and another in E2-pseudogene. For identification of three haplotypes, Hap_1 Hap_2 and Hap_3, primer-sets used were 14F+PS2R, 14F+PS1R and 24F+PS2R, respectively. The PCR reaction mixture (15 ul) contained 1X buffer with 1.5 mM MgCl2, 200uM each dNTP, 0.2 uM each primers and 0.5 units of taq polymerse. The PCR conditions were an initial denaturation at 95ºC for 3 minutes, followed by 35 cycles each comprising of denaturation at 95ºC for 0.30 s, annealing at 55ºC for 0.30 s, denaturation at 72º C for 1 min, and final extension at 72ºC for 7 min. Cloned products of each representative haplotype were used as a positive control.

3.2.5 Identification of gene duplication organization and breakpoint

Based on the fact that at least three haplotypes are present in all individuals from a DDT-resistant mosquito colony, we assumed that these haplotypes are actually paralog variants. To identify breakpoints, we designed variant-specific primers in both directions (forward and reverse) for two polymorphic loci--one in GSTe2 coding region (E21F, E21R, E22F and E22R) and another in the e2-psedogene (PS1F, PS1R, PS2F, PS2R), where indels of 12 bp and 23 bp, respectively, were recorded. A series

of PCR were performed using combinations as -forward+reverse, forward+forward and reverse+reverse of these primers pairs. In addition, primers, which are specific to GSTe1 (E1F), GSTe5 (E5R) and insert segment (ISF and ISR), were also used in various combinations. The list of primers and their sequences are shown in Table 3.2. For all these PCRs, long-PCR amplifications were carried out on gDNA of DDT-resistant and susceptible mosquitoes. The PCR reaction contained 1X LongAmp Taq reaction buffer (New England BioLabs), 2.5 units of LongAmp Taq DNA, 300 µM dNTPs, 0.1 µM of each primers and 0.25 µl of DNA in a total reaction volume of 25 µl. The thermal cycling conditions were: initial denaturation at 94ºC for 3 minutes followed by 20 cycles each of denaturation at 94ºC for 30S, annealing for 30C at temperature starting from 65ºC with increment of -0.5C each cycle and extension for 8 minutes at 72ºC. Remaining 15 cycles the annealing temperature was kept constant at 55ºC. The PCR products of successful PCR and with single band were purified using Exo-Sap before sequencing. Sequencing of PCR products was successfully carried out at Macrogen Inc using primer-walking strategy.

3.2.6 Quantitative PCR

Quantitative PCR (qPCR) was performed using the LightCycler® 480 Real-Time PCR Instrument (Roche Diagnostics Corporation). Each qPCR reaction (20 ul) contained 0.05 um primers and 1X SYBR® green real-time PCR master mix (Toboyo Co., Ltd, Japan) and 0.25 ul of DNA template. qPCR cycling conditions were: initial denature at 94°C for 5 mins, followed 40 cycles each with denaturation at 94°C for 15 s, annealing at 57°C for 20 s and extension at 72°C for 30 seconds, followed by dissociation curve analysis, which is by performing a temperature ramp for one cycle at 95°C for 10 s, 65°C for 1 min and gradual increase to 95 °C at 2°C per sec to check the specificity of PCR. Primers used are shown in Table 3.2. A total of 15 mosquitoes from the DDT susceptible strain and eight mosquitoes from the DDT-resistant line were analysed for relative copy number of GSTe2 with S7 ribosomal gene using qPCR method described above. Analysis of results for AsGST epsilon genes to understand duplication events was carried out specifically.

3.3 Results

3.3.1 Polymorphism in AsGST epsilon 2 and 4 genes

DNA sequencing of 20 wild-caught mosquitoes for AsGSTe2 and 15 mosquitoes for AsGSTe4 revealed the presence of four isoforms of AsGSTe2 and at two isoforms of AsGSTe4. The sequences of different variant forms and deduced amino acids for these genes are shown in Table 3.1. Among the four isoforms of AsGSTe2 named AsGSTe2.1, GSTe2.2, GSTEe2.3 and AsGSTe2.4, the first two are the most abundant and the latter two were rare and were found only in two mosquitoes. All isoforms contained 224 amino acids apart from AsGSTe2.2, which contained an additional 4 amino acids. The addition of four amino acids in AsGSTe2.2 is due to incorporation of repeat of six amino acids (Ser- Tyr –Iso- Ser- Ser- Iso) and deletion of two amino acids Val at positions 172 and 177 (Table 3.1). Cloning and sequencing of cDNA from a pool of ten mosquitoes confirmed amino acid sequence AsGSTe2.1 and AsGSTe2.2 (Appendix 3 &4).

Table 3.1. Synonymous and non-synonymous polymorphism in AsGSTe2 and AsGSTe4.

Isoforms	Codon position											
	164	165	166	167	168	169	172	173	174	175	176	177
	S	T	I	S	S	I	V	-	-	-	-	V
AsGSTe2.1	TCC	ACG	ATC	TCT	AGC	ATT	GTT	---	---	---	---	GTT
	S	T	I	S	S	I	**S**	**T**	**I**	**S**	**S**	**I**
AsGste2.2	TCC	ACG	ATC	TCT	AGC	ATT	TCC	ACG	ATC	TCT	AGC	ATT

Isoforms	Codon position					
	003	046	089	209	213	217
	R	E	S	A	L	E
AsGSTe4.1	AGG	GAG	TCC	GCA	CTC	GAA
	K	E	**R**	A	L	E
AsGSTe4.2	AAG	GAA	GCC	GCA	CTC	GAA
	K	E	**R**	A	**R**	E
AsGSTe4.3	AAG	GAA	GCC	GCG	CGT	GAG

Multiple evidences enumerated below confirm the presence of gene duplication event of a segment of GST epsilon array.

3.3.2 Unequal copy number of two AsGSTe2 isoforms in heterozygotes

One of the two isoforms, AsGSTe2.2 (Hap3), was difficult to detect in DNA sequence chromatogram because it was always present along with AsGSTe2.1 (Hap1 and 2) in low copy number, which resulted in extremely low peak height of AsGSTe2.2 as compared to dominant isoform AsGSTe2.1 (Figure 3.1A). AsGSTe2 with additional 12 bp can be detected in sequence chromatogram due to overlapping peaks from the start of the mismatch point. Alternatively we used a qPCR assay to detect these isoforms. The qPCR assay was highly specific due to incorporation of more than a six bp mismatch in the allele-specific primer at 3 prime end. The qPCR further confirms low copy number of AsGSTe2.2 as compared to AsGSTe2.1 (Figure 3.1 B)

3.3.3 Presence of more than two haplotypes in an individual

DNA sequencing of 20 individual clones of a PCR amplicon derived from a single mosquito encompassing partial AsGSTe2, e2-pseudogene and partial AsGSTe4 revealed the presence of three haplotypes. The sequences of three haplotypes (Hap1, Hap2 and Hap3) are as shown where Hap1 and Hap3 differ by a contiguous 16 bp nucleotide sequence in the coding region of AsGSTe2 resulting from a 12bp indel and 4 bp substitution and Hap2 differs from Hap1 throughout e2-pseudogene by SNPs and indels. Out of 20 clones sequenced, eight were Hap1, 10 were Hap2 and two were Hap3.

3.3.4 Total heterozygosity in a laboratory mosquito colony

In the event of gene duplication, there may be high frequency of heterozygosity or complete heterozygosity of paralogous alleles, which are located at different loci. We genotyped 21 individuals from laboratory-bred DDT-resistant *An. stephensi* for two polymorphic loci. One was in the coding region of AsGSTe2 and another present in the intergenic region (pseudogene) between AsGSTe2 and AsGSTe4. The primers designed for the AsGSTe2 locus were specific to Hap1 and Hap2+3 and the primers designed from the pseudogene were specific to Hap1+2 and Hap3 (Figure 3.2). All the allele-specific primers had a minimum six bp mismatch eliminating the possibility of mispriming. All the mosquitoes genotyped were positive for all the three haplotypes

(Figure 3.4) in each individual. Total heterozygosity strongly suggests that the three haplotypes are present at different locus indicating presence of gene duplication event.

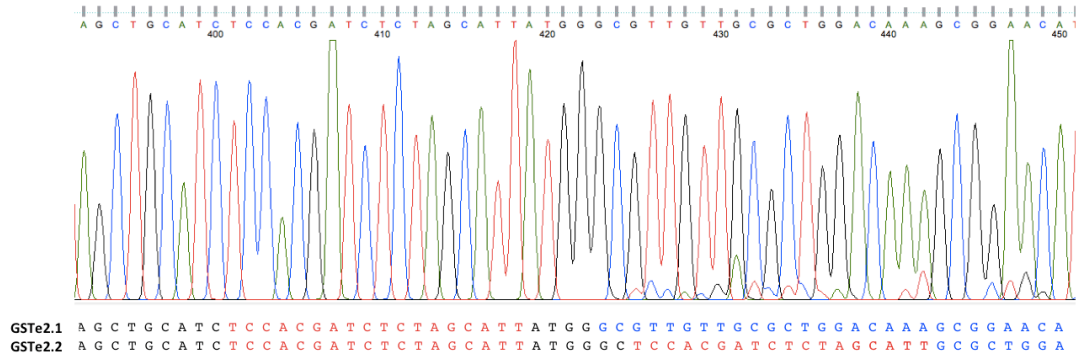


Figure 3.1. A. Unequal copy number of two GSTe2 variants (GSTe2.1 and GSTe2.2) in DDT-resistant strain: DNA sequence chromatogram showing unequal peak heights of GSTe2.1 and GSTe2.2 in heterozygote. The deduced nucleotide sequence of two variants has been shown at the bottom of chromatogram.

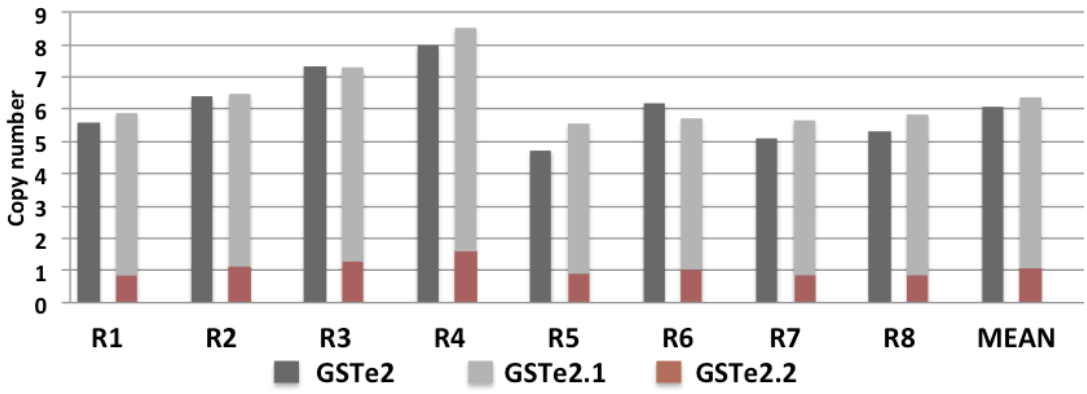


Figure 3.1.B. Estimated copy number of GSTe2 (total) and variants GSTe2.1 and GSTe2.2 as compared to ribosomal S7 gene in individual mosquitoes as determined by real-time PCR.

3.3.5 Pattern of gene duplication and identification of breakpoint

The organization of gene duplication based on the most likely arrangement through manual annotation of DNA sequences obtained from a series of long-PCRs is presented in Figure 3.5. This is the most plausible arrangement of gene duplication

showing a minimum of five tandem duplication in mosquitoes sequenced. It is notable that in the case of the presence of a tandem duplication of identical repeat units in the same direction, PCR will tend to amplify the shortest fragment and one repeat unit may be missed. The list of the primer sets used for PCR and product sizes are summarized in Table 3.2

It was observed that there are at least five tandem repeats of a 3.7 kb unit consisting of a complete AsGSTe2, e2-pseudogene and AsGSTe4 separated by a highly conserved 2,423 bp insert segment. All the duplicated segments were in the same direction and we failed to recognize any inverted gene duplication as all attempts to amplify PCR using same direction primers (forward-forward or reverse-reverse) resulted in no amplification. The breakpoint of all the duplicated events were identical, having one AsGSTe1 gene in one end and other in AsGSTe5. The duplicated segment consists of an array of complete AsGSTe2, e2-psedogene, AsGSTe4 and an intragenic spacer connecting them to partial AsGSTe1 (91 bp) at the 5' end and AsGSTe5 (156 bp) toward 3'end. The 2.7 kb intervening duplicated segment is designated as the Insert Segment (IS) as this is not homologous to GST array. VectorBase BLAST revealed that major portion of this (2,367 bp) is homologous to an unrelated scaffold (Supercontig KB665332).Careful examination of DNA sequence revealed that the IS non-coding and is not a transposon.

3.3.6 Genomic copy number variations in individuals as detected by qPCR

The relative mean number of copies of AsGSTe2 with reference to S7-ribosomal gene present in DDT susceptible and DDT-resistant line is shown in Figure 3.3. A significantly high copy number of AsGSTe2 was observed in DDT resistant line as compared to susceptible strain (1.7 vs 6.1; $p<0.0001$, t-test). AsGSTe2 showed differences in copy numbers between the DDT resistant and the susceptible strain, with the DDT strain having copy numbers ranging from 5-7 copies compared to the susceptible, , which has 1 to 2 copies in total

Figure 3.2. Schematic representation of the PCR strategy and the primer locations along the target fragments.

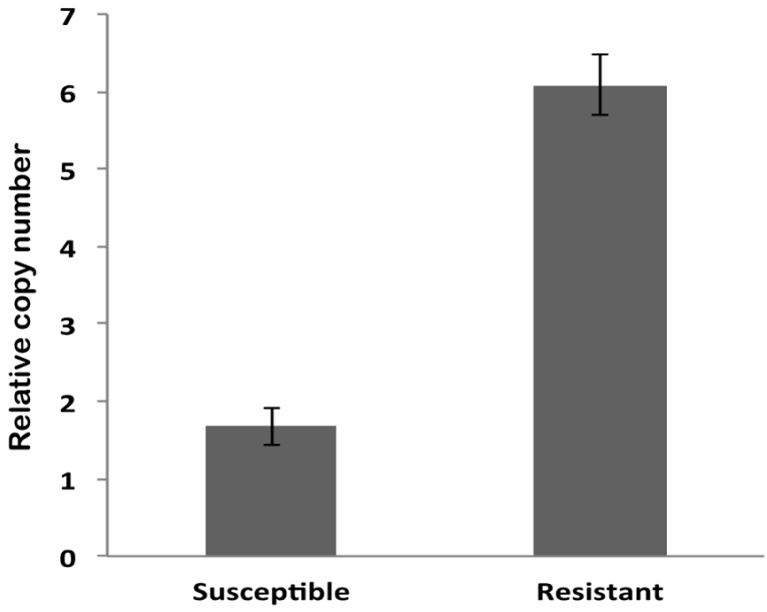
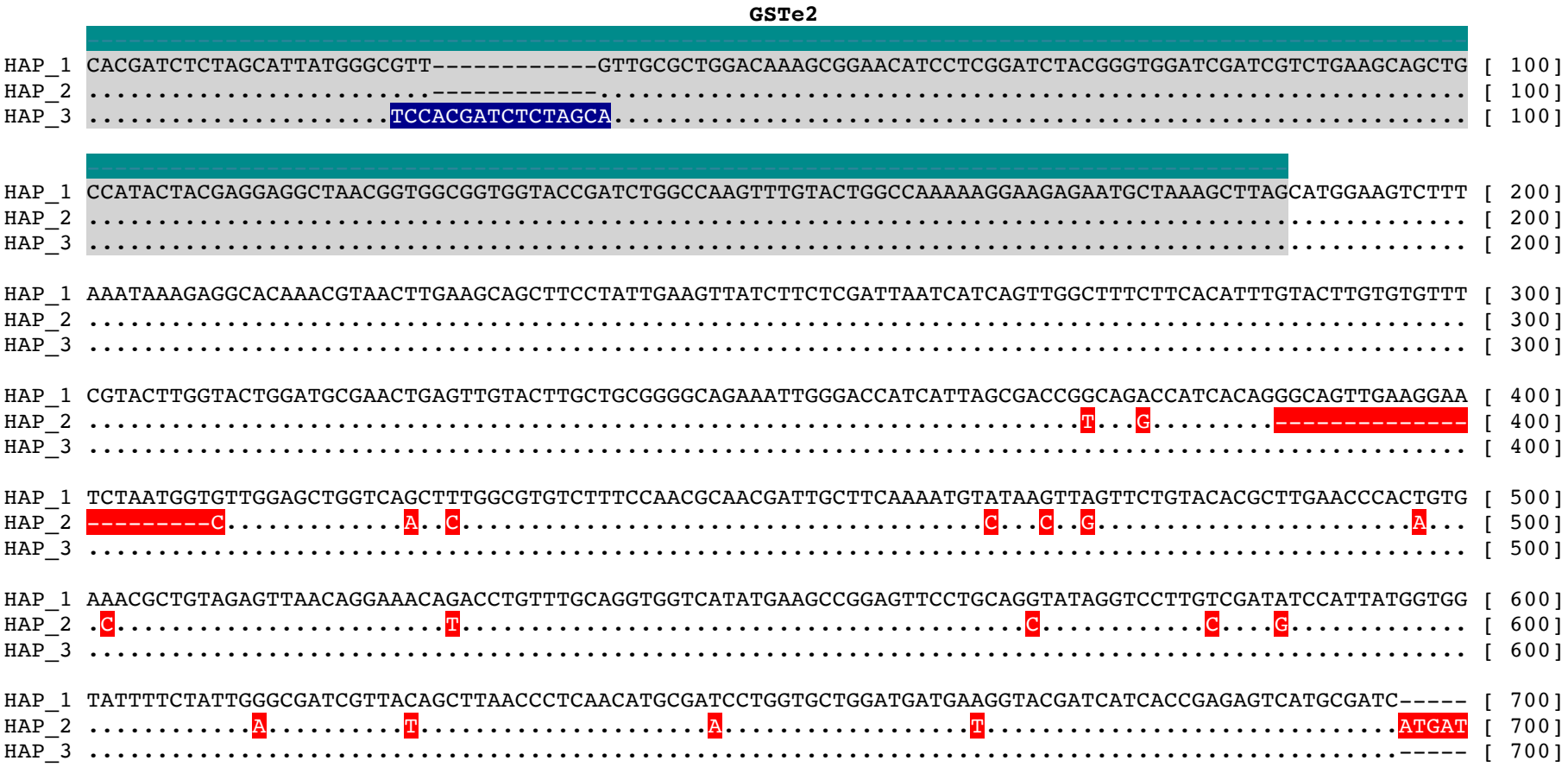
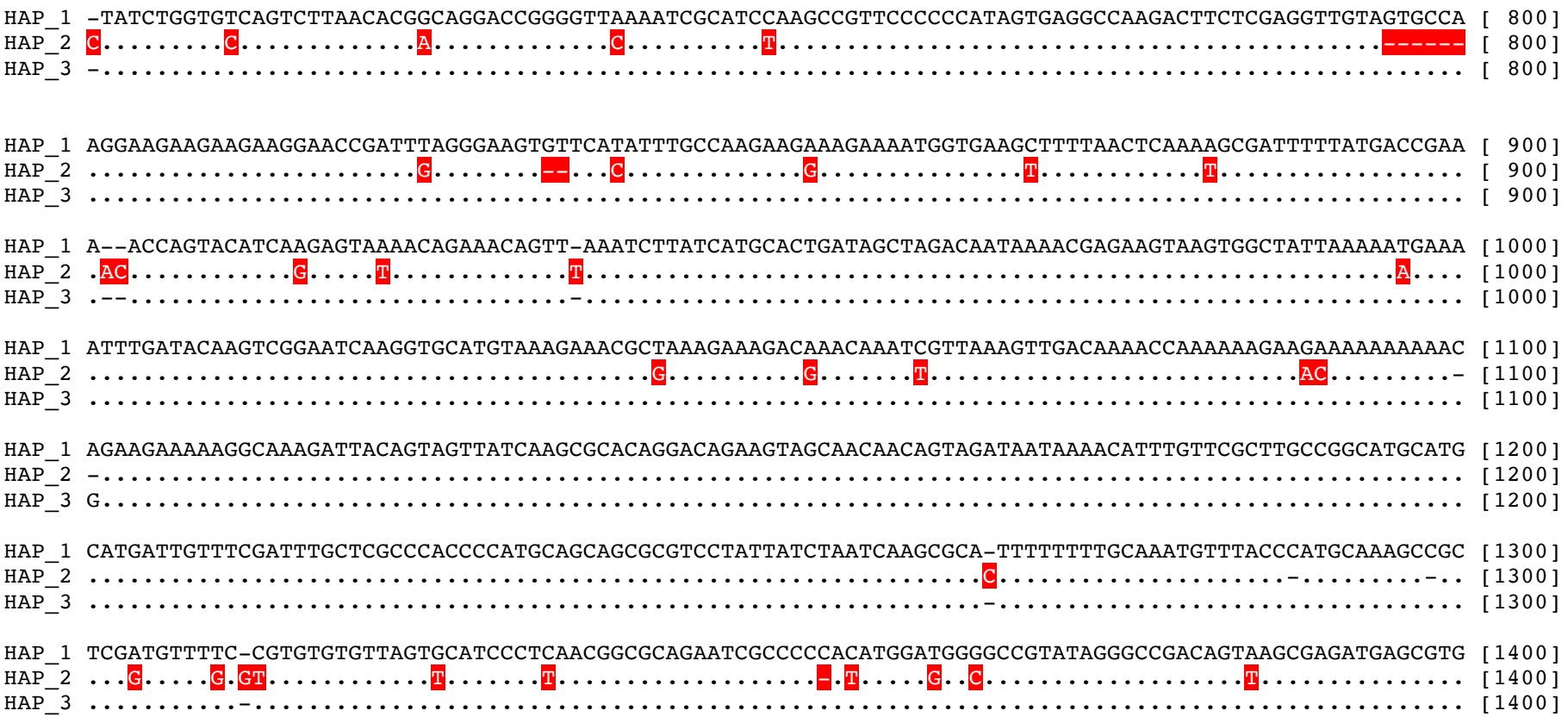


Figure 3.3. Mean relative copy number of GSTe2 in DDT-susceptible and -resistant *An. stephensi* with reference to housekeeping ribosomal S7 gene as estimated by qPCR. Error bar represents standard error, ($p<0.0001$; t-test) Standard deviation (SD) and standard error (SE) of susceptible and Resistant being 0.94, 0.24 and 1.12, 0.40 respectively.

Table 3.2. List of primers used in the study.

Name of primer	Location and specificity	Sequence (5′—3′)
qPCR primers		
E2F	GSTe2	GCC GAC TTT AGC TGC ATC TC
E2R	GSTe2	TCT CTT CCT TTT TGG CCA GTA
E21F	GSTe2.1	TCT AGC ATT ATG GGC GTT GTT G
E21R	GSTe2.1	CTT TGT CCA GCG CAA CAA C
E22F	GSTe2.2	CTA GCA TTA TGG GCT CCA CG
E22R	GSTe2.2	CTT TGT CCA GCG CAA TGC T
St_S7F	S7	TGC GTG AAT TGG AGA AGA AGT T
St_S7R	S7	CAG GAT GGC ATC GTA CAC AG
Amplification primers for DNA sequencing		
E1F	Intergenic spacer upstream to GSTe1	TCA GTT CAT TGC CGA CTT TG
E21F	GSTe2.1	TCT AGC ATT ATG GGC GTT GTT G
E21R	GSTe2.1	CTT TGT CCA GCG CAA CAA C
E22F	GSTe2.2	CTA GCA TTA TGG GCT CCA CG
E22R	GSTe2.2	CTT TGT CCA GCG CAA TGC T
PS1F	e2-pseudogene (type1)	GTC AGG CCA TCA CAG CTT G
PS1R	e2-pseudogene (type1)	TTG ACC AGC TCC AAG CTG TG
PS2F	e2-pseudogene (type2)	CAG ACC ATC ACA GGG CAG T
PS2R	e2-pseudogene (type2)	CTG ACC AGC TCC AAC ACC AT
PSR	GSTe4	CGA TCA GAT TGA TGG GCA CG
ISF	Insert segment	CCG AAT ACG GTA AGC TCC AA
ISR	Insert segment	CGT CGT GTG CTA AAC GAT GA
E5R	GSTe5	CCT GGT TGA GCT GCT CGT A


GSTe2
HAP_1 CACGATCTCTAGCATTATGGGCGTT----------GTTGCGCTGGACAAAGCGGAACATCCTCGGATCTACGGGTGGATCGATCGTCTGAAGCAGCTG [100]
HAP_2 .....----------..... [100]
HAP_3 .....TCCACGATCTCTAGCA..... [100]
HAP_1 CCATACTACGAGGAGGCTAACGGTGGCGGTGGTACCGATCTGGCCAAGTTTGTACTGGCCAAAAAGGAAGAGAATGCTAAAGCTTAGCATGGAAGTCTTT [200]
HAP_2 ..... [200]
HAP_3 ..... [200]
HAP_1 AAATAAAGAGGCACAAACGTAACTTGAAGCAGCTTCCTATTGAAGTTATCTTCTCGATTAATCATCAGTTGGCTTTCTTCACATTTGTACTTGTGTGTTT [300]
HAP_2 ..... [300]
HAP_3 ..... [300]
HAP_1 CGTACTTGGTACTGGATGCGAACTGAGTTGTACTTGCTGCGGGGCAGAAATTGGGACCATCATTAGCGACCGGCAGACCATCACAGGGCAGTTGAAGGAA [400]
HAP_2 .....T...G.....---------- [400]
HAP_3 ..... [400]
HAP_1 TCTAATGGTGTTGGAGCTGGTCAGCTTTGGCGTGTCTTTCCAACGCAACGATTGCTTCAAAATGTATAAGTTAGTTCTGTACACGCTTGAACCCACTGTG [500]
HAP_2 ---------C.....A..C.....C...C..G.....A... [500]
HAP_3 ..... [500]
HAP_1 AAACGCTGTAGAGTTAACAGGAAACAGACCTGTTTGCAGGTGGTCATATGAAGCCGGAGTTCCTGCAGGTATAGGTCCTTGTCGATATCCATTATGGTGG [600]
HAP_2 .C.....T.....C.....C....G..... [600]
HAP_3 ..... [600]
HAP_1 TATTTTCTATTGGGCGATCGTTACAGCTTAACCCTCAACATGCGATCCTGGTGCTGGATGATGAAGGTACGATCATCACCGAGAGTCATGCGATC----- [700]
HAP_2 .....A.....T.....A.....T.....ATGAT [700]
HAP_3 .....----- [700]


HAP_1 -TATCTGGTGTCAGTCTTAACACGGCAGGACCGGGGTTAAAATCGCATCCAAGCCGTTCCCCCCATAGTGAGGCCAAGACTTCTCGAGGTTGTAGTGCCA [800]
HAP_2 C.....C.....A.....C.....T.....------ [800]
HAP_3 -..... [800]
HAP_1 AGGAAGAAGAAGAAGGAACCGATTTAGGGAAGTGTTCATATTTGCCAAGAAGAAAGAAAATGGTGAAGCTTTTAACTCAAAAGCGATTTTTATGACCGAA [900]
HAP_2 .....G.....--...C.....G.....T.....T..... [900]
HAP_3 ..... [900]
HAP_1 A--ACCAGTACATCAAGAGTAAAACAGAAACAGTT-AAATCTTATCATGCACTGATAGCTAGACAATAAAACGAGAAGTAAGTGGCTATTAAAAATGAAA [1000]
HAP_2 .AC.....G.....T.....T.....A.... [1000]
HAP_3 .--.....-..... [1000]
HAP_1 ATTTGATACAAGTCGGAATCAAGGTGCATGTAAAGAAACGCTAAAGAAAGACAAACAAATCGTTAAAGTTGACAAAACCAAAAAAGAAGAAAAAAAAAAC [1100]
HAP_2 .....G.....G.....T.....AC.....- [1100]
HAP_3 ..... [1100]
HAP_1 AGAAGAAAAAGGCAAAGATTACAGTAGTTATCAAGCGCACAGGACAGAAGTAGCAACAACAGTAGATAATAAAACATTTGTTCGCTTGCCGGCATGCATG [1200]
HAP_2 -..... [1200]
HAP_3 G..... [1200]
HAP_1 CATGATTGTTTCGATTTGCTCGCCCACCCCATGCAGCAGCGCGTCCTATTATCTAATCAAGCGCA-TTTTTTTTGCAAATGTTTACCCATGCAAAGCCGC [1300]
HAP_2 .....C.....-.....-.. [1300]
HAP_3 .....-..... [1300]
HAP_1 TCGATGTTTTC-CGTGTGTGTTAGTGCATCCCTCAACGGCGCAGAATCGCCCCCACATGGATGGGGCCGTATAGGGCCGACAGTAAGCGAGATGAGCGTG [1400]
HAP_2 ...G.....G.GT.....T.....T.....-.T.....G..C.....T..... [1400]
HAP_3 .....-..... [1400]

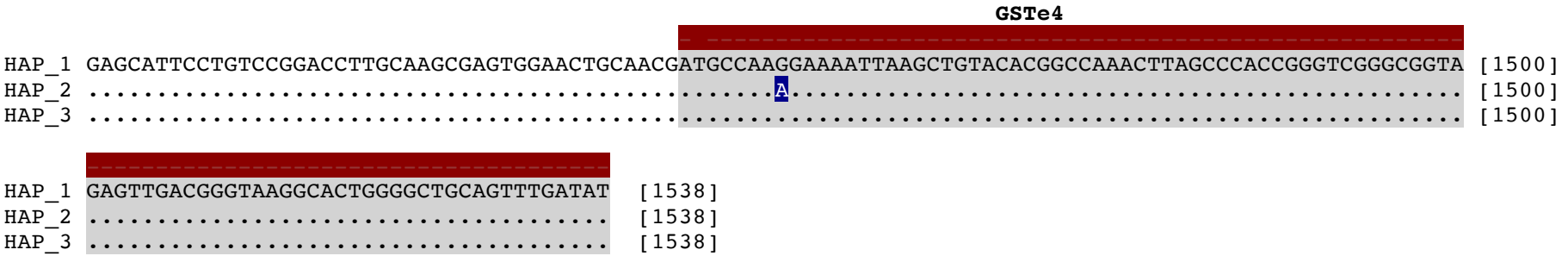


Figure 3.4. Haplotypes identified in *An. stephensi* epsilon GSTe2- GSTe4 array revealed through cloning.

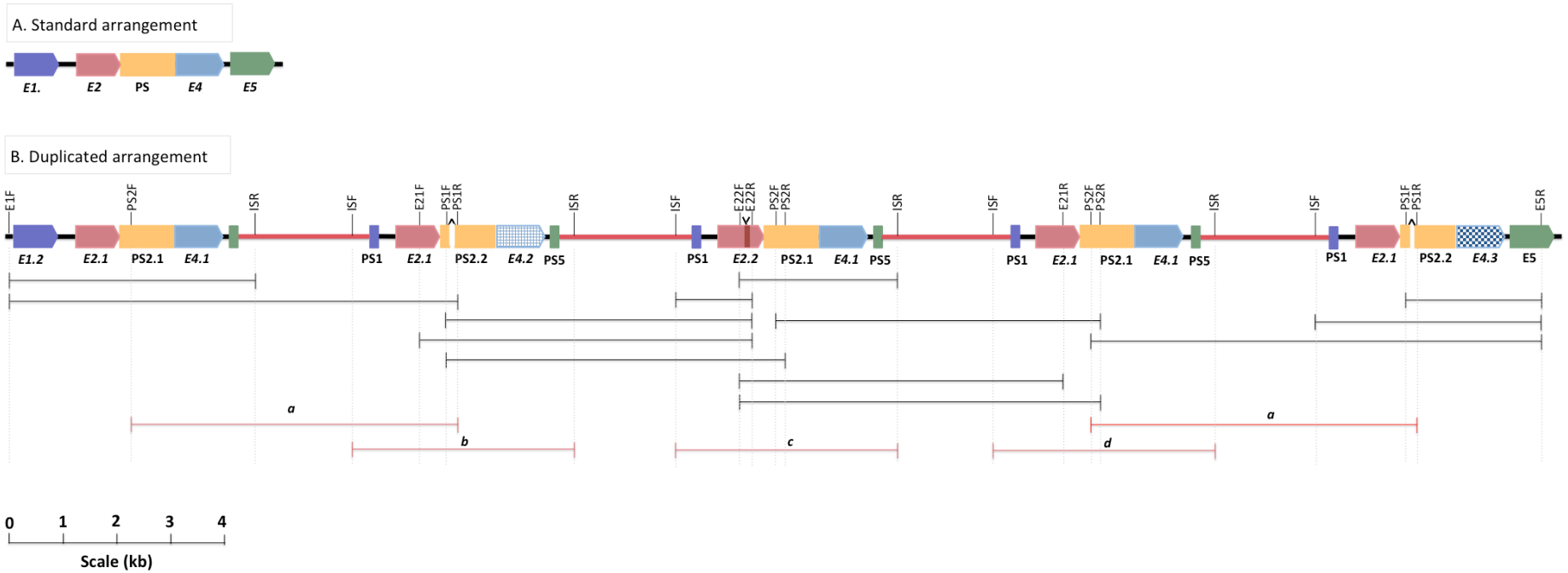


Figure 3.5. Arrangement of GST epsilon genes in *An. stephensi* (A) Standard arrangement of partial GST-epsilon gene array (Ayres *et al.* 2011). (B) Most plausible annotated arrangement of GST epsilon genes and gene duplication constructed on the basis of PCR amplicon size and DNA sequences. The coloured blocks are genes (including the pseudogenes). The black and red lines connecting genes are intergenic regions and insert region from unrelated part of genome, respectively. Location of primers have been shown in vertical text and successful PCR have been indicated by distance bars. The black distance bar represent successfully sequenced amplicons. Horizontal dimension lines indicate amplicon size derived with specific primer set Red horizontal dimension lines in each row represents PCR amplicon amplified by single PCR, which matches to two (denoted as 'a') or three locus (b, c and d) where 'a' are identical haplotypes and b, c and d are three haplotypes, which were phased out by sequencing with primers designed from variable region. Abbrev: E1 and E1.2: GSTe1; E2.1, E2.2: GSTe2; E4.1, 4.2, 4.3: GSTe4; PS2.1, 2.2: pseudogene GSTe2; PS1: partially duplicated GSTe1 PS5: partially duplicated GSTe5; E5: GSTe5.

3.4 Discussion

The data presented in the present study show evidence of gene duplication in the GST epsilon cluster AsGSTe2 and AsGSTe4 of the DDT resistant laboratory strain of *An. stephensi*. Evidence based on DNA sequencing and qPCR suggesting duplication of AsGSTe2 and AsGSTe4 include copy number variation of variants (marked by disproportionate peak heights in sequencing chromatograms and validated by quantitative PCR), presence of more than 2 haplotypes in a single individual, and permanent heterozygosity (three haplotype combination), which is characteristic of all samples arising from a uniform strain. Thus, indicating multiple loci for a particular polymorphic allele. DNA sequencing of PCR product using primers from regions with indels showed tandem duplication of AsGSTe2 and AsGSTe4 in multiple copies where each repeat unit separated by an insert segment of 2.7 kb, which is unrelated to the GST epsilon cluster array.

Glutathione-*S*-transferases are known to have a role in detoxification of DDT in many insects. The mechanism generally being the quantitative increase in enzyme expression and DDT dechlorinase activity. Recently it has been demonstrated that a qualitative change driven by a single mutation L119F in the GSTe2 DDT resistant gene is associated with higher metabolic activity in the DDT resistant *An. funestus,* with the ability to metabolize the pyrethroid permethrin where significant permethrin metabolism was observed for the Benin 119 F-GSTe2 allele (45% depletion) (Riveron *et al.,* 2014). In our study, there is high expression of the DDT resistance implicated AsGSTe2 gene but no occurrence of the L119F mutation is noted in the *An. stephensi* samples examined. However tandem co-duplication of AsGSTe4 and AsGSTe2 is observed each encoding a portion of AsGSTe1, AsGSTe2, Ase2-pseudogene, AsGSTe4 and partial AsGSTe5, intervened by an insertion segment (IS). The IS and the e2 pseudogene are noncoding DNA and these differed greatly in terms of nucleotide diversity as IS was found to be highly conserved while GSTe2 pseudogenes had a number of SNPs. The GST gene family like other multigene families such as P450s is widely accepted to have evolved through successive gene duplications events (Soranzo *et al.,* 2004).The arrangement of duplication observed in this gene has not be

reported in this gene family in insects. In total, two isoforms of AsGSTe2 and three isoforms of AsGSTe4 were found present in an individual mosquito.

Gene duplication supplies raw material for functional novelty of genes at the genotype level (Conant *et al.,* 2008), where each duplicated copy then evolves independently diversifying the effects (Bridges, 1935). Functional differences between duplicated genes can be from mutations that are directly responsible for new functions, subdivision of ancestral functions or selection for changes in gene dosage. Genomic evidence in yeast (Qian *et al.,* 2014) showed the definite role gene duplication plays in adaptation by organisms to a challenging environment (antibiotic resistance), which is important for evolutionary innovation. Recent duplication and copy number polymorphism has been argued as a plausible mechanism of genomic adaptation by an organism to a stressful or novel environment (Kondrashov *et al.,* 2012). The retention of the different isoforms of AsGSTe4, which are functional as evident from their enzyme activities with different substrates discussed in chapter 4 maybe an indicator that they have an important role from a functional and an evolutionary point of view although their metabolic role in DDT resistance is ruled out.

Copy number variation in a gene allows for diversification of the repertoire of novel genes created in response to rapidly changing environments (Iskow *et al.*, 2012). Conceptually, fitness of a duplication event in a gene explains the dosage response where increase in copy number leads to an increase in protein dosage (Kondrashov *et al.,* 2012). In the present study, DDT resistant strains have significantly higher DNA copy numbers compared to its susceptible counterpart. Further, duplications with emphasis on their derived amplifications are due to multistep processes, which frequently happen under the force of positive selection for the increased copy number and such duplications arise at different timescales and through distinct mechanisms (Ream *et al.,* 2015). *Anopheles stephensi* GSTe4 association with AsGSTe2, evident in this study, may play a role in secondary metabolism via glutathione conjugation. As yet the functional relationship of AsGSTe4 with DDT resistance is unclear. It may also be possible that AsGSTe4 is under positive selection through association with AsGSTe2 or the co-expression is due to shared transcription factors or rather an integrative regulatory system as evident in yeast (Tsai *et al.,* 2007), which allows

yeasts to simultaneously modulate expression of neighboring genes in order to adapt to changing environments rapidly and efficiently.

Diversification of gene families is widely attributed to the phenomenon of successive gene duplication, which is a major driving force in evolution. Our data suggests that a gene duplication event in *An. stephensi* has led to the diversification of two functional variants of AsGSTe2 and three variants of AsGSTe4. The two variants of AsGSTe2 are due to a deletion of 12 bp and an insertion of 4 bp in the AsGSTe2 gene. On the other hand the three variants of AsGSTe4 are due to non-synonymous SNPs at the positions 3, 89 and 213 of the amino acid residues Arginine, Serine and Lysine respectively where AsGSTe4.2 differs from AsGSTe4.1 by SNPs at position 3 and 89 and the third AsGSTe4.3 varies from the two by an additional SNP at position 213. Besides the non-synonymous mutations happening in the gene, several synonymous mutations were recorded to accumulate alongside, which leaves us with a hypothesis that the present gene duplication in the AsGSTe gene has not occurred recently. The annotated five repetitive units of the duplication show divergence, with the components--e2 pseudo gene i.e., PS1 and PS2 being highly diverse. Beside divergence in repetitive units, the position of breakpoint and sequence of IS is conserved indicating that IS is not neutral but may be playing some regulatory function.

Several examples of duplication events associated with insecticide resistance have been reported in insect resistant genes. The first of examples are seen in the amplification of the esterase in *Culex* mosquito ((Mouches *et al.,* 1986) and green peach aphid, *Myzus persicae* (Field *et al.,* 1988), then in organophosphate (OP)-resistant sheep blow flies, where esterase E7 gene is duplicated simultaneously to maintain two different resistance alleles on two different loci (Campbell *et al.,* 1997, 1998) and in the 9 fold amplification Cyp6CY3 in *Myzus persicae* that is associated with neonicotinoid resistance (Li *et al.,* 2007) although the mechanism has not been elucidated. Again, tandem duplication of CYP6p9 and CYP6p4 with 21 and 25 times overexpression in resistant female *An. funestus*, suggests that P450 gene duplication may have a role in pyrethroid resistance by increasing pyrethroid metabolism (Wondji *et al.*, 2009). Duplication of the *Rdl* (GABA) receptor subunit gene in *M. persicae* is

one possible cause for the resistance to cyclodiene insecticides (Anthony *et al.,* 1998) and in drosophila where 4000 fold resistance is due to Ala (301) to Ser mutation, and the individuals bearing one WT copy of *Rdl* and a second copy with two point mutations: an Ala (301) to Ser resistance mutation and Met (360) exhibit intermediate dieldrin resistance (Remnant *et al.,* 2013). The *Ace*-1 in gambiae is duplicated where additional copies of the resistant *ace* -1 G119S alleles confer survival advantage to carbamate resistance (Edi *et al.,* 2014). Duplication led to the existence of two *ace*-1 loci encoding both resistant and susceptible (Bourguet *et al.,* 1996). The dual copies of acetylcholinesterase (AChE) play a role in resistance and adaptation in Culex mosquitoes reducing the fitness cost associated with the mutant ace allele. Multiple copies of it were confirmed to confer resistance to an OP insecticide in the two-spotted spider mite (Kwon *et al.,* 2010). Comparative analysis of GSTs in the red flour beetle, *T. castaneum*, using bioinformatics reference tools reported an inverted long-fragment duplication in the genome besides, it showed that most of the GSTs are tandemly arranged in three chromosomes with the epsilon (Shi *et al.,* 2012) although no functional relation was attempted. Most detoxification genes such as esterase and CYP undergo extensive gene amplification to heighten gene dosage, as evident. In our study, it is can be inferred that the duplication event of the resistance related AsGSTe2 gene has a role in DDT resistance.

Mechanisms of gene duplication often remain uncertain. Several proposed mechanisms aimed at assessing duplication rates and dependencies considering factors such as turn -over rates of such events, which is hard to measure besides, how each duplication mechanism works within a genome often varies with its position. (Reams *et al.,* 2015) It can be mentioned the mechanism of duplication evident in the AsGST epsilon stands unclear at this stage and a comprehensive understanding on its relation or evolving regulation involved in conferring DDT resistance is to be properly developed before a conclusion is definite. We were unable to correlate the number of copies of AsGSTe2 in field population with DDT resistance but qPCR performed on laboratory selected DDT resistant strain had significantly higher copy number (mean >6) as compared to susceptible strain. Such findings suggest that gene duplication may

be playing a role in resistance against DDT rather than just an overexpression of AsGSTe2.

3.5 Conclusion

Tandem co-duplication of partial GST epsilon covering AsGSTe2 and AsGSTe4 has been identified in the DDT resistant line of *An. stephensi*. The higher genomic copy number of AsGSTe2 in DDT resistant strain as compared to susceptible suggest gene duplication may enhance DDT metabolism and thus associated with DDT resistance and may likely be an adaptive mechanism in response to the insecticide toxicity.

Chapter 4. EXPRESSION AND CHARACTERISATION OF RECOMBINANT DETOXIFICATION ENZYMES GLUTATHIONE-S-TRANSFERASE EPSILON 2 AND 4 IN *ANOPHELES STEPHENSI*

Abstract

Transcriptional profile of the GST epsilon genes revealed AsGSTe2 and AsGSTe4 to exhibit elevated expression in DDT resistant *An. stephensi* laboratory strains compared to the susceptible strain. Glutathione-*S*-transferase epsilon AsGSTe2 and AsGSTe4 variants of the Indian urban malaria vector *An. stephensi* were expressed and characterized. Enzyme characterization of two of the predominant recombinant variants AsGSTe2.1 and AsGSTe2.2 showed significant DDT dehydrochlorinase activity. *Anopheles stephensi* GSTe4 did not metabolize DDT suggesting no direct role in the first phase DDT metabolism. Thermostability tests recorded AsGSTe2 unlike its orthologues, to be highly unstable at higher temperature. In- silico analysis and molecular docking of AsGSTe2 variants showed that none of the variant amino acids lie in the DDT binding site suggesting they have may have no association with DDT resistance in this vector.

4.1 Introduction

Anopheles stephensi a malaria vector in urban India responsible for 12% of the malaria cases (Dash *et al*., 2007) has reportedly manifested high resistance to DDT (Tikar *et al*., 2011). Ranson and Hemingway (2005) associated increased levels of Glutathione S-transferases (GSTs) with organochlorine and organophosphate insecticide in insects particularly in mosquitoes. Glutathione S-transferase epsilon 2 (GSTe2) gene was identified and found associated with the major DDT resistance locus, rtd1 (Ortelli *et al*., 2003). Fivefold expression of GSTe2 in DDT resistant strains with high DDTase activity in recombinants, and later in a majority of insect species, defined the role of GSTe2 in DDT metabolism (Ranson *et al*., 2001). Another report showed GST to offer passive protection against pyrethroids by binding to their molecule in a sequestering mechanism (Kostaropoulos *et al*., 2001).

Belonging to a family of multifunctional intracellular enzymes, GSTs act to detoxify via glutathione conjugation, dehydrochlorination, glutathione peroxidase (GPx)

activity or passive/sacrificial binding (Hayes and Wolf 1988, Mannervik and Danielson 1988, Pickett and Lu 1989, Yang *et al*., 2001). Reduced glutathione (GSH) conjugates with compounds having electrophilic centers through the formation of a thio-ether bond between the sulphur atom of GSH and the substrate, converting a reactive lipophilic molecule into a water soluble, non-reactive excretable conjugate (Habig *et al*., 1974). Of the three GST protein families (microsomal, cytosolic and mitochondrial) in eukaryotes, the cytosolic family is implicated in resistance (Hemingway *et al*., 2004). GST classes, alpha, mu, pi, theta, sigma, beta, chi, omega, zeta are based on amino acid sequence similarity, immunological, kinetic, and tertiary/quaternary structural properties. Within a class, cytosolic GSTs share >40% identity whereas only approximately 25% identity exists between classes (Sheehan *et al*., 2001). GST functions in detoxification of both endogenous and xenobiotic compounds either by direct action on these compounds or by catalyzing the secondary metabolism of a vast array of compounds that are oxidized by cytochrome P450 family (Ranson and Hemmingway, 2005).

Mitchell *et al*, 2014 described how an allelic variant of GSTe2, 114T in concert with another allele variant1014F of the voltage gated sodium channel (VGSC) is associated with DDT resistance in Ghana (Mitchell *et al*., 2014). Riveron, in 2014 demonstrated that a mutation in the GSTe2 increases the enzyme function in the biodegradation of DDT in resistant mosquitoes when compared to susceptible strains (Riveron *et al*., 2014). Characterization of GSTe2 variants in DDT resistant and susceptible strains arising from differences at 5 residues revealed the resistant isoform to exhibit a higher affinity for the insecticide DDT although having comparable DDT dehydrochlorinase activity (Lumjuan *et al*., 2011).

Considering the continuing widespread resistance to DDT, which has witnessed extensive use in India, a better understanding of GST would be an added advantage for an early and effective detection in insecticide resistance management. This chapter centered its objective on identifying and characterizing the possible role of GST epsilon with DDT resistance in *An. stephensi*, an urban malaria vector in India, drawing out a comparative baseline on the enzymatic activity of the identified recombinants. Evidence of overexpression of AsGSTe2 and AsGSTe4 in a DDT-resistant *An. stephensi colony* maintained in the laboratory, with polymorphisms existing at the transcript level of these two genes in wild caught and resistant

mosquitoes led to the study. Here, we characterized the recombinant enzymes arising from two transcripts of AsGSTe2 and three of AsGSTe4. The AsGSTe2 and AsGSTe4 isozymes were characterized to determine if they can metabolize DDT efficiently, as confirmed in other insects' GST epsilon. Besides, protein thermostability comparison was also drawn out with GSTe4 and GSTe2 orthologues of *Aedes aegypti* and the African vector *An. gambiae*. *In-silico* molecular modelling and structural analyses were attempted to rationalize structural aspects of DDT binding or enhanced enzyme activity played by the amino acids polymorphisms.

4.2 Material and methods

4.2.1 Mosquito collection and colonisation

Anopheles stephensi mosquitoes were collected from National Capital region and Alwar district, (Rajasthan) in 2012 and from Chennai (Tamil Nadu) in 2014-2015. As described earlier, mosquitoes were identified in the laboratory using a light dissecting microscope following Christopher's key (Christopher, 1933). Two to three day old female F1 mosquitoes were reared and maintained in the laboratory maintaining temperature at 25±3o C, 70-85% RH, and monitored dawn and dusk (12 hours). DDT and deltamethrin resistant mosquitoes were obtained by exposing field collect to DDT and deltamethrin. Laboratory selection through intermittent exposure of DDT for succeeding generation was performed periodically to build up resistance.

4.2.2 Insecticidal Bioassay

WHO's standard susceptibility tests were carried out on F1 to discriminate DDT - resistant and susceptible mosquitoes as described earlier. Care has been taken to maintain the environmental temperature at the recommended $27^{o}C \pm 2$ (WHO, 1998) when carrying out susceptibility tests using pyrethroids since temperature is found to play a fundamental role in determining the outcome of insecticide exposure in an array of insect species (Glunt *et al*., 2013). Following assays a portion of the surviving mosquitoes were preserved in RNA later and stored in $-80^{o}C$for experiments on RNA. Meanwhile progeny for the two strains were expanded for downstream experiments.

4.2.3 Quantitative enzyme assay

For enzyme assays, DDT and the deltamethrin resistant strains of *An. stephensi* and the susceptible strain were used for enzyme assays as per WHO protocol. It is to be noted that mosquitoes for enzyme assays were not to be pre-exposed to DDT or deltamethrin. Mosquitoes were individually homogenized in micro centrifuge tubes containing 200 µl assay buffer as final volume placed in a -20º C cryo-box. The lysate was centrifuged at 14,000 RPM for 30 seconds and the supernatant was used as enzyme samples in the case of GST. In case of other enzyme assays, the lysate was aliquoted for different enzyme assays used for carrying out enzyme kinetic, which were attempted preferably in triplicates. The remaining unused lysate is then stored at -80ºC immediately until use.

4.2.4 RNA extraction and reverse transcription

Total RNA from *An. stephensi* was extracted using TRIzol reagent (Invitrogen, CA, USA) and All DNA/RNA microprep (Qiagen, Hilden, Germany), combining both protocols following the manufacturer's instruction with slight modification, which includes the incubation of the to be extracted RNA with isopropanol at -20ºC overnight followed by transferring of the RNA-isopropanol mix into an RNA mini elute column for RNA purification and proceeded as per manufacturer's instructions in the Qiagen RNA microprep . Reverse transcription (RT PCR) of the mRNA was achieved using Revert Aid double strand cDNA synthesis kit (Fermentas, Thermo Fisher) following the manufacturer's protocol and the resulting cDNA was either directly used for further PCR amplification or quantitative PCR (qPCR), else it is stored at – 20ºC in aliquots until use. Each aliquot is intended for single use.

4.2.5 Quantitative expression of *An. stephensi* GST epsilon

Gene specific primers for GST epsilon genes were designed to examine the expression profile of all GST epsilon genes in *An. stephensi* using quantitative PCRs. Protocols were standardized with primer efficiency tested for each primer pair and expression profiling was carried out on DDT-resistant and susceptible mosquitoes. Primers pairs for GST epsilon (1-7) were designed for real time experiments (Table 4.1). Length of amplicon of each gene was targeted between 70 to 110 mers.

Table 4.1. Primer sequence of primers used in qPCR of the AsGST epsilon.

Primer	Sequence 5'-3'
GSTE1F	TGATGGGACAATCATCGCCGAA
GSTE1R	GCGAAGAACACGAGTTCCGT
GSTE2F	TGCACTTCGAATCCGGTGTACT
GSTE2R	GTTTTTCCGTAGAACAGAATACGTTCAA
GSTE4F	ACCGAGAGCCACGCAATCAT
GSTE4R	GCCGAAGTACAGAATAGGTTCCAA
GSTE5F	AAAAGTAAACGCCGGTCTGCAC
GSTE5R	CCTTCGTACAGGATCGGTTCGA
GSTE6F	ATGTCTAGCAAGCCGGTCCTAT
GSTE6R	CCCTTGAAGACGTTCATCTCCCTAAT
GSTE7F	TGAATCCGCAGCAAACGATACC
GSTE7R	TGCCCGAATAAATTACCGGTTCAA

4.2.6 Amplification, cloning and sequencing of *An. stephensi* GSTE2 and GSTe4 genes

Glutathione S transferase epsilon specific primers were designed for amplification of the full coding region of GST epsilon gene with the help of GST epsilon reference genes downloaded from *An. stephensi* genome database available from VectorBase. Initial amplification of the GST epsilon genes AsGSTe2 and AsGSTe4 was carried out by using flanking primers GSTe2F1 (5'-ACGAGCGCAAGTGAAATCAT-3') and GSTe2R1 (5'-ACGTTTGTGCTTCTTTATTTAA-3') for AsGSTe2 and GSTe4F (5'-CTTGCAAGCGAGTGGAACT -3') and GSTe4R (5'-CTTTGGGCTTTGAGCGTAGT-3') for AsGSTe4 designed respectively. Internal Primers GSTe2F2 5'-CTCCAACGACCACAATCATG-3' and GSTe2R2 5'-TGCTTCAAGTTACGTTTGTGC-3' were designed for nested approach. For further

downstream amplification of epsilon AsGSTe2 and AsGSTe4, leading to protein expression, primers pETE2F (5'-CTA GCT AGC ATG CCT AAG CTA GTT CTG TAC-3') and pETE2R (5'-CCG CTC GAG CTA AGC TTT AGC ATT CTC TTC-3') and pETE4F (5-CTA GCT AGC ATG CCA AAG AAA ATT AAG CTG TAC-3') and pETE4R (5'- CCG CTC GAG TCA CTT GGC TTT GGC ACG AT -3') were designed respectively where a six-base pair tail (CTA GCT) was added to the 5' end to create restriction site (RE sites) for downstream protein expression. GST epsilon 2 and 4 were amplified separately using 2ul cDNA in a 25ul reaction using Phusion High Fidelity DNA Polymerase (NEB, Ipswich, Massachusetts, USA) containing 1X buffer, 1.25 uM of each primer and 2U taq. The PCR conditions were an initial denaturation of 98ºC for 20 secs and a 30 cycle denaturation at 98ºC for 10 secs, annealing at 60ºC for 15 secs and extension at 68ºC for 5 mins in ABI 9700 thermal cycler. Amplified product was cloned using CloneJET PCR cloning kit (Fermentas, Thermo Fisher) and sequenced at Macrogen Inc, South Korea.

4.2.7 Construction of a prokaryotic expression plasmid

Genomic DNA was isolated using DNA isolation kit (Qiagen, USA). AsGSTe2 and AsGSTe4 gene was amplified using Phusion High-Fidelity DNA Polymerase (New England Biolabs, UK) using 5′- CTA GCT AGC ATG CCT AAG CTA GTT CTG TAC -3′ and 5′- CCG CTC GAG CTA AGC TTT AGC ATT CTC TTC-3′ and 5′- CTA GCT AGC ATG CCA AAG AAA ATT AAG CTG TAC -3′ and 5′- CCG CTC GAG TCA CTT GGC TTT GGC ACG AT -3′ as forward and reverse primers respectively. The restriction sites consist of NheI i.e., G^CTAGC and XhoI i.e., C^TCGAG for the forward and reverse primers respectively. The PCR involved 30 cycles of denaturation at 98 °C for 20 s, annealing at 66 °C for 15 s followed by elongation at 72 °C for 15 s. This plasmid product and was digested and cloned into already digested and purified Pet28a+ vector. The clone was verified by sequencing. After sequencing, the correctly cloned plasmid and pET28a vector were both digested by NheI and XhoI restriction enzymes. The GSTe gene fragment and the linear plasmid were recycled after agarose electrophoresis; connected by T4 DNA ligase to construct the recombinant expression plasmid pET28a-AsGSTe. The plasmid was transformed into *E. coli* DH5α competent cells and positive clones were screened following, which the correct pet28a-GSTE clone was transformed in BL21 DE3 competent cells for protein expression.

4.2.8 Chemicals and kits

Biochemical reagents were procured from Sigma–Aldrich Chemical Company, St. Louis, MO, USA while protein purification kits, Ni–NTA agarose were purchased from Qiagen, CA, USA and GE Healthcare Life Sciences UK. Restriction enzymes and were procured from New England Biolabs, MA, USA. Other reagents such bacterial culture media was from Invivogen and Clonetech. Buffer solutions 14.3 M 2-Mercaptoethanol,2M imidazole pH8,1M Tris –HCL pH 8.5, 5M NaCl, Lysis buffer, Ni-NTA wash buffer, Elution buffer, 2X storage buffer, 0.1M potassium phosphate buffer pH6.5,75mM Glutathione (GSH) (Sigma G4251),30mM CDNB (Aldrich 23, 732-9) were prepared in-house.

4.2.9 Expression and purification of recombinant AsGSTe2 and ASGSTe4

A single transformant from the pET28a-BL21 (DE3) GST epsilon transformation was inoculated in a 5ml Lb broth with antibiotics- ampicillin (100 µg/mL) and kanamycin (50 µg/mL) culture overnight. This was sub cultured in a 800ml broth medium in a 2 litre flask and allowed to grow overnight at 37°C and 200 rpm. Expression of GST epsilon was induced with 0.3mM isopropyl-β-D-thiogalactoside (IPTG) at 16°C when an optical density (OD) of 0.6-0.8 at 600 nm was attained by the overnight culture. Resultant cells were harvested by centrifuging at 4500g for 15 minutes at 4^0C and then resuspended in 10ml lysis buffer (25 mM TrisHCl pH 8.5, 500 mM NaCl, 20 mM imidazole), Dnase1 and protease inhibitor EDTA and 5 mM β-mercaptoethanol and the pellet disrupted by sonication and then centrifuged at 17000rpm at 4^0 for 30 minutes to obtain a clear supernatant. The supernatant , which is the His-tagged GSTe2 is then carefully filtered and the purification carried out employing Immobilized metal ion affinity chromatography (IMAC) technique using Ni-NTA agarose affinity resin (Qiagen,Valencia, CA, USA) following the manufacturers' instructions. The resultant Ni-purified proteins were desalted using Amicon ultra – 15 centrifugal filter units 10K (GE Healthcare) and concentration of each protein determined and visualized on SDS page. The purified and quantified protein was reconstituted in 40% glycerol and stored in -80ºC for further use.

4.2.10 Protein assay

Quantification of total protein was done as per standard Bradford assay (Bradford, 1976) procedure in triplicates employing Bovine serum albumin of known concentration as a standard protein for quantifying unknown.

4.2.11 Western blotting

The protein cocktail His-tagged recombinant GSTe2 was analyzed on a 12.5 % SDS-PAGE and transferred to a nitrocellulose (Amersham Hybond ECL) for Western Blotting. Pre-synthesized peptide antibodies having the sequence QFVLSQKEKNAQKA to match the carboxy terminal of GSTe2 protein were used in western blotting. PBS having 2% Bovine serum albumin (BSA) was used for saturation after, which the membrane was then probed with pre-synthesized anti-GSTe2 polyclonal rabbit antibodies (dilution 1:10,000). Horseradish peroxidase labelled anti-rabbit antibody (dilution 1:50000; Santacruz biotechnology) was employed as secondary antibody following , which the binding was then visualized by using ECL Advanced blotting detection kit (G E Healthcare) /incubating the membrane with di-amino-benzidine (DAB) (sigma Germany) with a metal enhancer.

4.2.12 Glutathione-s- transferase enzyme activity

Glutathione (reduced) and CDNB as substrates were used to determine GST activity spectrophotometrically at 340 nm using the extinction coefficient for the product S–(2, 4–dinitrophenyl) glutathione (ε340 nm = 9.6 mM–1 cm–1). The assay mixture (311ul) comprised of GST enzyme and 75 mM GSH in 100 mM potassium phosphate buffer pH 6.0 containing 150 mM NaCl. Enzyme was replaced with distilled water in blank .The reaction was started by addition of 30 mM CDNB and reading was recorded for 3 minutes; the rate of reaction was linear with time during this period. One unit of GST activity was defined as the conjugation of 1 μmol of CDNB with GSH per minute at 25 °C. The data recorded for the rate (increase in conjugated product concentration) of GSH conjugation to CDNB is a measure of GST activity and this is assayed spectrophotometrically with a microplate reader FLUOstar Omega microplate reader (BMG Labtech) at 25ºC and 340 nm. The kinetics km and Vmax were studied for CDNB and DCNB Three replicates, each of varying concentration of CDNB (4mM-0.03125mM) keeping GSH concentration constant at 10mM were performed running three replicates each of varying concentration of CDNB (4mM to 0.03125mM)

keeping GSH concentration constant at 10mM. Vmax, the maximum enzyme rate where the active site of the enzyme is fully bound to the substrate, Kcat, which measures the overall catalytic reaction turn-over rate and the Km, the substrate concentration for GSTe2 equivalent to half its maximum velocity were derived from the equation. Analysis was done using Graphpad Prism v5.03 (GraphPad Prism software, San Diego, USA).

4.2.13 Thermostability tests

The effect of temperature on the enzymatic activity of *An. stephensi* GST epsilon 2 and GST epsilon 4 was measured by carrying out a series of enzyme activity assays, as previously carried out however keeping a temperature range between 0°C and 50°C. Briefly, the enzyme was incubated with 30mM CDNB and 75mM GSH in triplicates for 5 mins at 7 different temperatures settings starting from 0°C, 4°C, 25°C, 30°C, 35°C, 40°C and 45°C to assess the thermostability property of the recombinants.

4.2.14 DDT dehydrochlorinase assay

DDT dehydrochlorinase activity of recombinant AsGSTe2 variants was assessed with reverse phased High Performance Liquid Chromatography (HPLC) silica based stationary phase, following Prapanthadara (1993) and Riveron (2014). DDT dilution series ranging from 200mg/ml to 3.125 mg/ml were prepared and reverse phase HPLC with optimized parameters is performed to separate DDT and DDE. 0.2 U of the recombinant enzyme was used in triplicates for the DDT concentration series. The amount of DDT in micrograms was calculated using the standard curve derived from the DDT peak area and then % depletion of DDT calculated accordingly. Retention time scale is scored between 5.23 - 5.50 mins for DDT and 5.75 - 6.05 mins for DDE. In brief, metabolic assays with DDT were prepared in 100mM potassium phosphate buffer (pH 6.5), 2.5 mM GSH and 0.2 units of individual GSTe2 and GSTe4 recombinant enzymes in 10ug/ml DDT in methanol, making sure the solvent is ≤ 10% of the total reaction volume (500ul) and maintaining controls with same constituent mix but a deactivated corresponding recombinant enzyme boiled prior to incubation. Incubation was done at 1200 rpm shaking for one hour at 25ºC -30ºC after, which 500µl of methanol was added to the reaction to bring it to a stop. The reaction mix was then centrifuged for 20 mins at 13000 rpm for 20 mins. 200µl of the supernatant was then transferred to HPLC vials and the amount of DDT and DDE, which remained in the reaction was assayed at an absorbance of 232nm using HPLC set up (Chromoleon,

Dionex, Sunny vale, CA, USA). A 100µl of each sample was injected into a 250mm C18 column (Acclaim120, Dionex, Sunnyvale, CA, US) at 23ºC. DDT and DDE were separated using an isocratic mobile phase of 93% acetonitrile and 7% water with a flow rate of 1 ml/min. Standard curves for DDT and DDE (double dilution series of 200- 12.5µg/ml) were obtained employing a non-linear regression fit and the results were analysed with the help of GraphPad Prism v5.03 software (GraphPad Software, Inc, San Diego, CA, USA).

4.2.15 AsGSTe2 protein modelling and docking with DDT

To generate the structural model of Glutathione S-transferase epsilon2 (GSTe2) transcripts by homology modeling method, the template crystal structure was searched in PDB database (Berman *et al*., 2000; Johnson *et al*., 2008) by querying GSTe sequences using NCBI-BLAST online service (Altschul *et al*., 1990). Known structures of GSTe2 in *An. funestus* (PDB: 3ZML) (Riveron *et al*., 2014), *An. gambiae* (PDB: 2IL3) (Wang *et al*., 2008), and Zan/U variant of *An. gambiae* (PDB: 4GSN) (Mitchell *et al*., 2014) were selected as templates to model GSTe2 transcripts in *An. stephensi.* Since, reduced glutathione is necessary for activation of GST, its coordinates were introduced from *An. gambiae* glutathione S-transferase epsilon 2 template (PDB: 2IL3). Subsequently, 20 models of each GSTs transcript were generated using Modeller package (Webb and Sali, 2014). These models were scored using Z-DOPE value (Shen and Sali, 2006), further, top scorers were evaluated for stereo-chemical refinement by PROCHECK (Laskowski *et al*., 1993) and RMSD (Root mean square deviation). The best model from each transcript was selected for further studies. PASS (Brody *et al*., 2000) was used to find out the surface pockets of the modeled proteins and the G-site (an active site for reduced glutathione, GSH) and H-site (an active site for DDT) of GSTe2 transcripts were predicted. The 3D structure of DDT was obtained from the Cambridge Structure Database (Ref. Code: CPTCEL). Autodock Vina was used to perform all docking simulations (Trott *et al*., 2009). Proteins and ligand were prepared by adding polar hydrogen atoms, and assigning Gasteiger atomic charge (Ramachandran *et al*., 1963) using AutoDockTools (Morris *et al*., 2009). In addition, hydrogen atoms were added to the GSH molecule and cysteine SH group was deprotonated to obtain GS^- for the docking calculations. The docking space was defined by placing a box of the 22.5 Å x 22.5 Å x 22.5 Å around the coordinates (center x=23.65, center y = -18.67, center z = 61.55), that covers active

sites (DDT and GSH binding site). The flexible residues were assigned to allow freedom of movement in sidechain rotamers (Leu 8, Leu 10, Ser 11, Leu 35, His 40, Arg 111, Phe 114, Gle 115, Leu 118, Phe 119, Leu 206 and Phe 209). The docking simulations were performed by considering an enhanced exhaustiveness of 32 to maximize the probability of finding global minimum conformation, and 10 binding poses for each model structures were calculated. The best pose from each docking simulation was selected on the basis of energy score.

4.3 Results

4.3.1 Quantitative PCR of AsGSTe genes

Comparative qPCR of the GST epsilon cluster of *An. stephensi* from a susceptible strain and the highly resistant DDT strain revealed an overall relative expression at higher levels by AsGSTe2 and AsGSTe4 in the DDT resistant strain as illustrated in Figure 4.1. AsGSTe7 showed little elevation compared to AsGSTe5 and AsGSTe6, while AsGSTe1 showed downregulation compared to the rest in the epsilon group. AsGSTe8 and ASGSTe3 were not attempted.

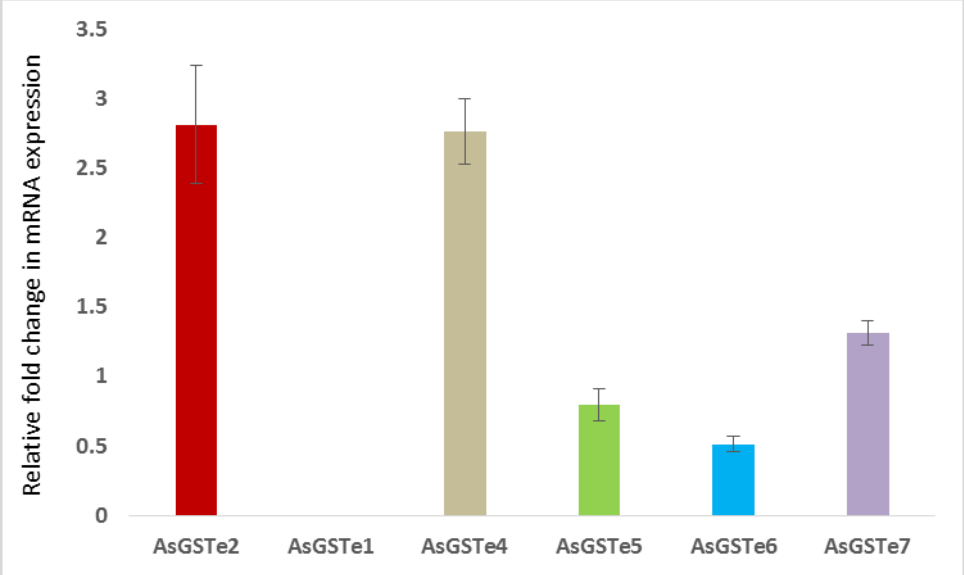


Figure 4.1. Relative fold change in mRNA expression of the GST epsilon cluster in DDT resistant and susceptible *An. stephensi* strains. Error bar denotes the SD of Δct from three replicates. (AsGSTe2 $p<0.0004$; AsGSTe4 $p<0.0001$; AsGSTe5 $p<0.005$; AsGSTe6 $p<0.0002$; AsGSTe7 $p<0.0004$; t-tests).

GSTe2 from a susceptible and DDT resistant strain of *An. stephensi* were examined for differences in gene expression using quantitative PCR. Results revealed a fivefold increase in expression of AsGSTe2 in the DDT resistant laboratory strain relative to

the susceptible *An. stephensi* as illustrated in Figure 4.2, suggestive of its role in conferring resistance against DDT in this vector.

4.3.2 AsGSTe2 and AsGSTe4 DNA copy number and gene expression

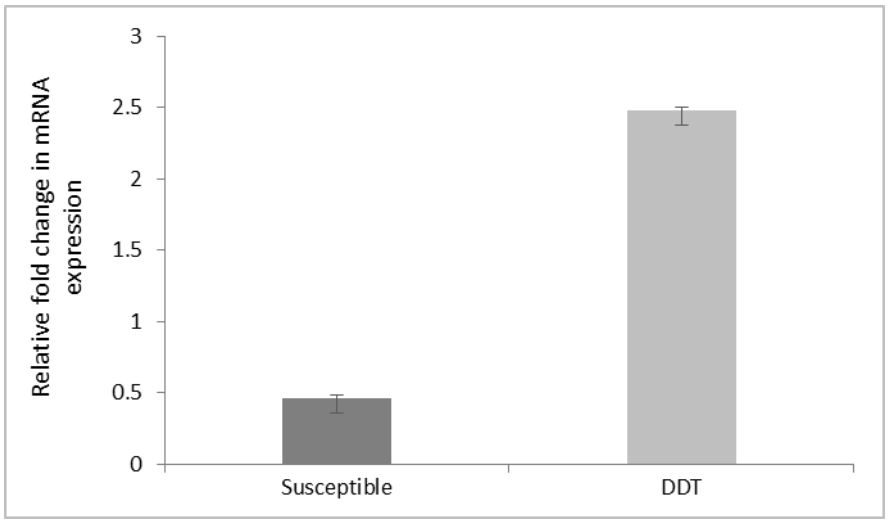


Figure 4.2. Relative expression of the GST epsilon 2 in DDT resistant and susceptible *An. stephensi* strains with reference to ribosomal S7.Error bar denotes SEM; ($p<0.007$; t-test).

DNA sequences of the variants of two epsilon genes GSTe2 and GSTe4 in *An. stephensi* are provided in Figure 4.3. Quantitative PCR showed AsGSTe2.1 to occur at higher copy number in DDT resistant mosquitoes from the lab as well and is also observed in the DDT resistant field samples tested (limited numbers), where a relative mean copy number ranges from 5-7 copies compared to the susceptible strain which showed a total of 1 or 2 copies only. While much of the work relating to duplication in *An.stephensi* has been carried out using gDNA, mRNA amplification with regard to gene expression is unclear although it is assumed that the copies should be translated to mRNA. It is interesting to note the existing changes in the promoter region of the different genes in the AsGSTe cluster which are changed by the duplication process. Such can also lead to altered gene expression. The limitation of the present study is a lack of work relating to RNA expression in relation to duplication and DDT resistance in *An. stephensi.* The other variants AsGSTe2.3 and AsGSTe2.4 were found in a very limited number of mosquitoes.


M P K L V L Y T L H L S P P C R A V E L T A K A L G [26]
AsGSTe2_2 ATG CCT AAG CTA GTT CTG TAC ACG CTG CAC TTG AGC CCA CCG TGC CGG GCT GTG GAA CTG ACG GCC AAG GCG TTG GGA [78]
AsGSTe2_1 ... ... [78]
AsGSTe2_3 ... ...T ... ... [78]
AsGSTe2_4 ... ... [78]
L E L E Q K N I N L L A G D H L Q P E F L K L N P Q [52]
AsGSTe2_2 TTG GAG CTG GAG CAG AAG AAC ATC AAT CTG CTG GCC GGT GAC CAT TTG CAG CCG GAG TTC TTG AAG CTT AAC CCC CAA [156]
AsGSTe2_1 ... ... [156]
AsGSTe2_3 ... ...T ... [156]
AsGSTe2_4 ... ...T ... [156]
H T I P V L D D D G T I I T E S H A I M I Y L V T K [78]
AsGSTe2_2 CAT ACG ATC CCG GTG CTG GAT GAT GAT GGT ACG ATC ATT ACC GAG AGC CAT GCA ATC ATG ATC TAT CTG GTG ACG AAG [234]
AsGSTe2_1 ... ... [234]
AsGSTe2_3 ..C ... ...G ... ... [234]
AsGSTe2_4 ..C ... ...G ... ... [234]
Y G K D D S L Y P K D P V K Q A R V N A A L H F E S [104]
AsGSTe2_2 TAC GGC AAG GAT GAC TCC CTC TAC CCG AAG GAC CCA GTC AAG CAG GCT CGT GTG AAC GCT GCC CTG CAC TTC GAA TCC [312]
AsGSTe2_1 ... ... [312]
AsGSTe2_3 ... ... [312]
AsGSTe2_4 ... ... [312]
G V L F A R M R F I F E R I L F Y G K T D L P E D R [130]
AsGSTe2_2 GGT GTA CTG TTT GCC CGG ATG CGA TTC ATC TTT GAA CGT ATT CTG TTC TAC GGA AAA ACG GAC TTG CCC GAG GAT CGC [390]
AsGSTe2_1 ... ... [390]
AsGSTe2_3 ... ... [390]
AsGSTe2_4 ... ... [390]
V E Y V Q K S Y R L L E D T L L D D F/Y V A G P A M T [156]
AsGSTe2_2 GTT GAG TAT GTG CAG AAA TCG TAC CGC TTG CTG GAG GAC ACG CTG CTG GAC GAT TTT GTA GCC GGA CCG GCC ATG ACG [468]
AsGSTe2_1 ... ... [468]
AsGSTe2_3 ... ...A ... ... [468]
AsGSTe2_4 ... ...A ... ... .A. ... ... [468]
I A D F S C I S T I S S I M G S/V T/- I/- S/- S/- I/V A L D K A [182]
AsGSTe2_2 ATT GCC GAC TTT AGC TGC ATC TCC ACG ATC TCT ACC ATT ATG GGC TCC ACG ATC TCT ACC ATT GCG CTG GAC AAA GCG [546]
AsGSTe2_1 ... ... GTT --- --- --- --- G.. ... ... [534]
AsGSTe2_3 ... ... GTT --- --- --- --- G.. ... ... [534]
AsGSTe2_4 ... ... GTG --- --- --- --- G.. ... ... [534]
E H P R I Y G W I D R L K Q L P Y Y E T/I A N G G G G [208]
AsGSTe2_2 GAA CAT CCT CGG ATC TAC GGG TGG ATC GAT CGT CTG AAG CAG CTG CCA TAC TAC GAG GAG GCT AAC GGT GGC GGT GGT [624]
AsGSTe2_1 ... ... [612]
AsGSTe2_3 ... .C ... ...A ... ..T ... ..T ..A ... ... [612]
AsGSTe2_4 ... ... A.. ... ... [612]
TACGAG
T/A D L A K F V L A K K E E N A K A * [225]
AsGSTe2_2 ACC GAT CTG GCC AAG TTT GTA CTG GCC AAA AAG GAA GAG AAT GCT AAA GCT TAG [678]
AsGSTe2_1 ... ... [666]
AsGSTe2_3 G.. ... ... [666]
AsGSTe2_4 ... ... [666]

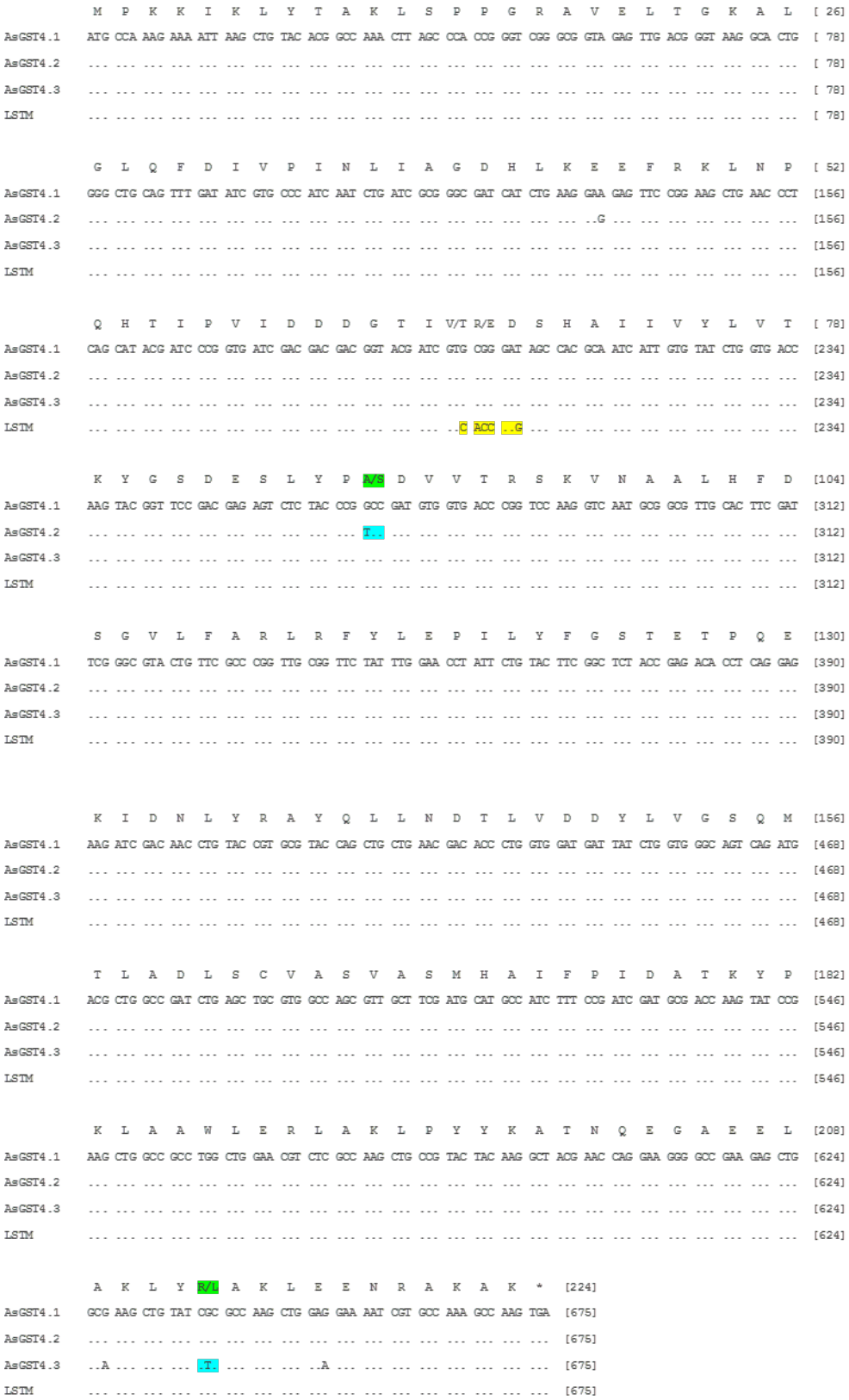


Figure 4.3. Polymorphism as seen in *An. stephensi* Glutathione S- transferase Epsilon 2 and Epsilon 4.

4.3.3 Recombinant protein expression

For expression of GSTe2 and 4 recombinants of *An. stephensi,* pET28a protein expression system was employed and is illustrated in Figure 4.4. Specifically designed primers (Table 4.2) were used for prior amplification of the genes.

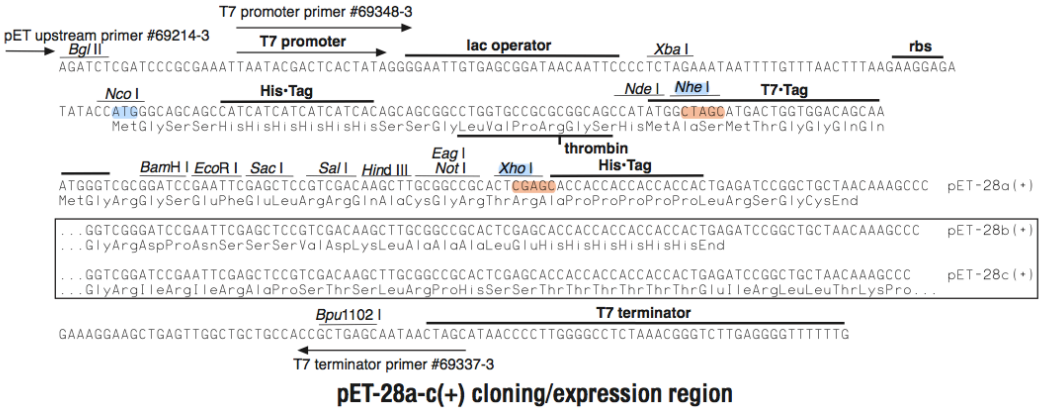


Figure 4.4. Vector map for Protein expression of AsGSTe2 and 4 recombinant.

Table 4.2. Specifically designed primers bearing restriction sites for protein expression.

Name	Sequence	Tm
pET_e2F	CTA GCT AGC ATG CCT AAG CTA GTT CTG TAC	57.5
pET_e4F	CTA GCT AGC ATG CCA AAG AAA ATT AAG CTG TAC	58.3
pET_e2R	CCG CTC GAG CTA AGC TTT AGC ATT CTC TTC	58.4
pET_e4R	CCG CTC GAG TCA CTT GGC TTT GGC ACG AT	58.4

AsGSTe2 and e4 recombinant proteins were successfully expressed in *E.coli*. A band of the expected size for GST, 25kDa, was evident in protein fractions from IPTG induced cultures.

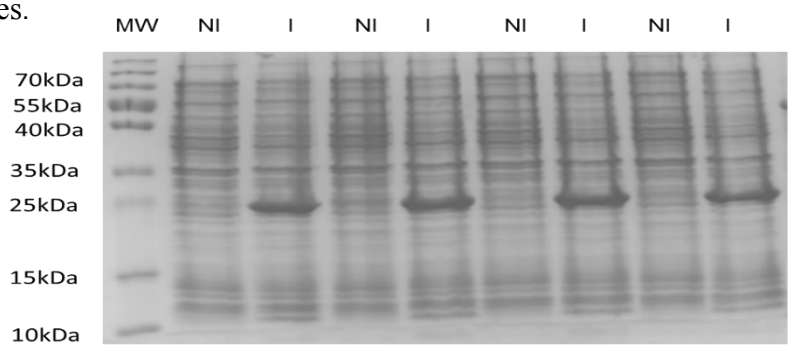


Figure 4.5. Denaturing gel photograph (SDS –Page) of protein fractions prior to purification. Abbreviations: MW= molecular weight, NI= non induced, I= Induced.

4.3.4 Protein purification

His tagged recombinant *An. stephensi* GSTe2 variants, AsGSTe2.1 and AsGSTe2.2 were purified using immobilized metal ion affinity chromatography (IMAC) Ni-NTA affinity resin. AsGSTe4 and AeGSTe2 were relatively pure (>90%), compared with AsGSTe2 variants, which contained several contaminating bands (produce a single composite Figure 4.6). The AsGSTe2 purification process was thus further optimized (Figure 4.7).

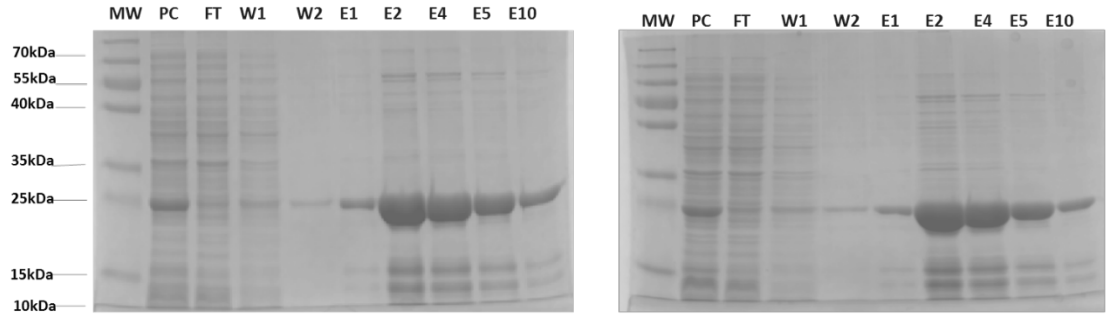


Figure 4.6. SDS page showing protein fractions of AsGSTe2 variants GSTe2.1 and GSTe2.2 using IMAC Ni-NTA affinity resin but showing non- specific banding below the desired band of 25kDa for GST. Abbreviations: MW=mol weight, PC=pre column, FT= flow through, W= wash, E= elution.

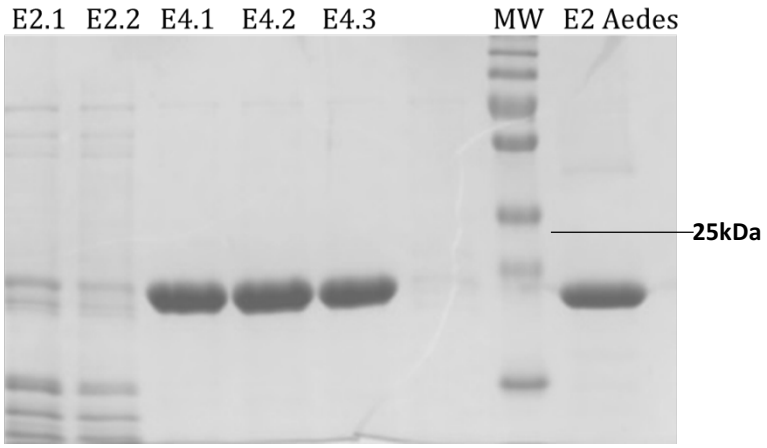


Figure 4.7. SDS page showing different purified protein variants of AsGSTe4 and AsGSTe2 at 25kDa; Aedes GSTe2 kept as a control. Abbreviations: E=elution fractions, M= molecular weight.

Western Blotting with his-tag antibodies suggested that low molecular weight bands were due to degradation of AsGSTe2 recombinants. Keeping the protein at 4^{o}C reduced degradation producing a single recombinant protein band (Figure 4.8)

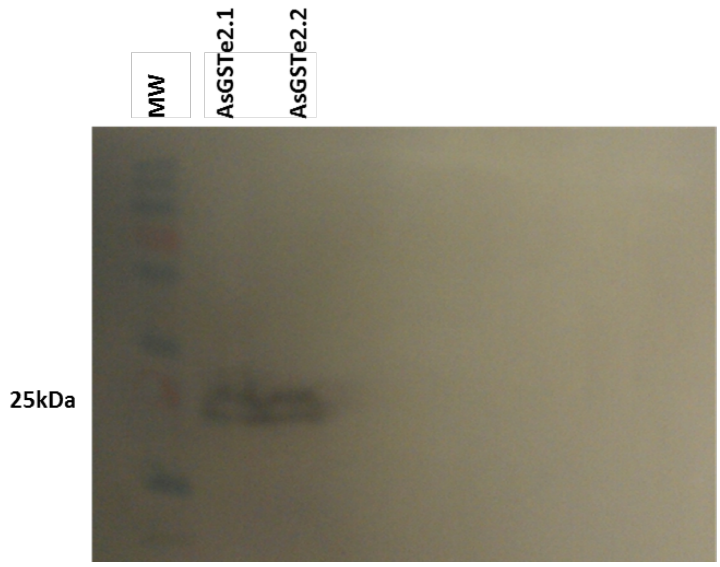


Figure 4.8. Western blotting on recombinant GSTe2 variants after optimization keeping at low temperatures of 4^0C or lower. Abbreviations: MW= mol wt; 1=AsGSTe2.1, 2= AsGSTe2.2.

4.3.5 Effect of temperature on enzyme activity (Thermostability)

Thermostability is an attribute of a protein by, which it resist any change in its structure due to heat application. The stability of AsGST epsilon proteins at a defined temperature was determined from the biological activity remaining after incubating the protein for 3 minutes at specific temperatures within a wide range. The effect of temperature on the residual enzymatic activity of the AsGSTe2 and AsGSTe4 was measured between 4°C and 45°C. Thermostability comparative experiment of the AsGSTe proteins is illustrated in Figure 4.9. It was shown that AsGSTe4 variants are more thermostable compared to AsGSTe2, with AsGSTe4.2 and AsGSTe4.3 exhibiting higher stability than the partially stable AeGSTe2, and AgGSTe2 where residual activity is observed at 40ºC , which then drops down sharply. It was observed that all other GSTe2 including *Ae. aegypti* and *An. gambiae* showed progressive loss of activity with increasing temperatures. Maximum residual activity of AsGSTe2 variants was maintained up until 20°C only with a sharp decline as room temperature is approached. While the thermo-instability observed in AeGSTe2 is said to be due to redundant cysteine residues present in *Aedes* besides the conserved residue found in *An. gambiae* and *An. funestus,* such is not the case in *An. stephensi.* The low thermostability in AsGSTe2 could be due to unforeseen issues related to optimization during expression and purification, which need to be ruled out.

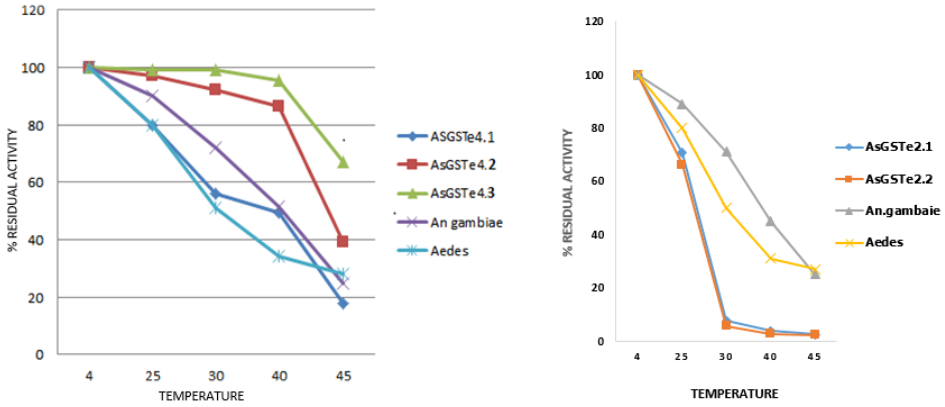


Figure 4.9. Thermostability of GST epsilon enzymes A) GSTe4 of *An. stephensi s.s* vs GSTe2 of *An. gambiae* and *Ae. aegypti*. b) GSTe4 of *An. stephensi.*

4.3.6 Specific activity and kinetic parameters of AsGSTe2 and ASGSTe4

The levels of GST activity associated with DDT resistance were assayed using GST model substrates 1-chloro-2,4-dinitrobenzene (CDNB), 3,4-dichloronitrobenzene (DCNB) and cumene hydroperoxide (CHP), with reduced glutathione (GSH) where the conjugate formation of substrates CDNB and DCNB with reduced glutathione (GSH) and GSH peroxidase activity with CHP were monitored. GSTe2 variants of *An. stephensi* showed similar CDNB specific activities to *Aedes aegypti* and *An. gambiae* variants (Table 4.3). No catalytic activity was detected against CHP. *As*GSTe4 variants not significantly diff from GSTe2 variants, in specific activity ~1.93 – 2.55 µmol/ min/ mg.

Table 4.3. Specific activity of recombinant AsGSTe2 and AsGSTe4 against model substrates.

	µmol/min/mg of protein		
Enzymes	CDNB	DCNB	CHP
AsGSTe2v.1	2.55	2.68	ND
AsGSTe2v.2	2.00	3.42	ND
AsGSTe4v.1	2.36	NT	NT
AsGSTe4v.2	2.59	NT	NT
AsGSTe4v.3	1.93	NT	NT
AgGSTe2	2.05	NT	NT
AeGSTe2	2.44	NT	NT

*ND= no enzyme activity detected; Ag-*gambiae*, Ae-*aedes,* As- *stephensi*, NT= not tested

Comparative kinetic analysis of AsGSTe2 variants is presented in Figure 4.10 using varying amounts of CDNB substrate concentrations with constant glutathione (GSH) concentration (Appendix 4).

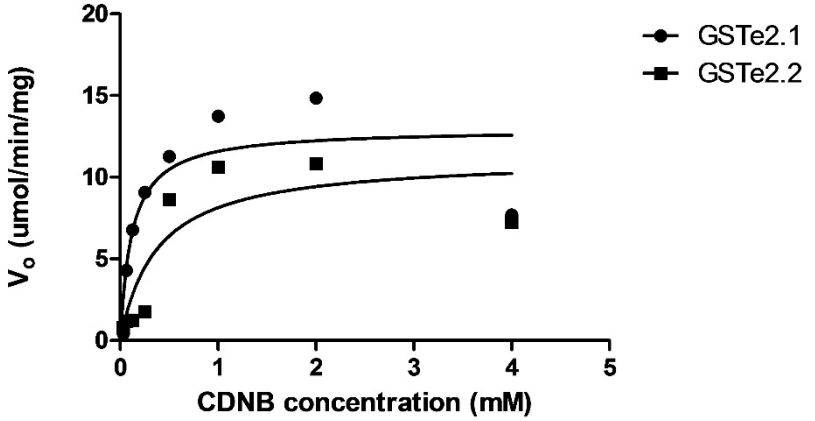


Figure 4.10. Kinetics of AsGSTe2 variants compared at varying concentrations of CDNB as a substrate.

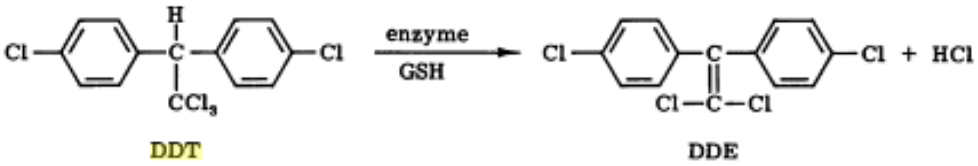
The enzyme activity of AsGSTe2 variants at different concentrations of substrate CDNB show highest activity of AsGSTe2.1 at 0.5mM CDNB and of AsGSTe2.2 at 4mM suggesting higher efficiency. V_{max}, the maximum enzyme rate where the active site of the enzyme is fully bound to the substrate, K_{cat}, which measures the overall catalytic reaction turn-over rate and the K_m, the substrate concentration for GSTe2 equivalent to half its maximum velocity were calculated and presented in Table 4.4.

Table 4.4. Kinetic parameters of recombinant GST epsilons in *An. stephensi.*

Enzyme	Vmax CDNB (µmol/min/mg)	K_{cat} (s^{-1})	K_m^{CDNB} (mM)	K_{cat}/km ^{CDNB} (mM^{-1} s^{-1})	Vmax DCNB (µmol/min/mg)	K_m^{DCNB} (mM)	K_{cat}/K_m ^{DCNB} (mM^{-1} s^{-1})
AsGSTe2.1	12.95 ± 1.79	8 ± 1.14	0.12 ± 0.07	66.66	2.83 ± 0.32	0.47 ± 0.14	3.8
AsGSTe2.2	11.26 ± 2.56	11 ± 2.41	0.39 ± 0.29	28.20	2.80 ± 0.41	1.15 ± 0.34	0.94

4.3.7 DDT dehydrochlorinase assay

DDT dehydrochlorination is catalyzed by the enzyme GSTe2 in the presence of glutathione (GSH) to give 1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethylene (DDE) (Ranson, 2001) represented in a simple chemical equation as



DDTase activity of AsGSTe2.1 and AsGSTe2.2 was examined and compared with AgGSTe2, which has known DDTase activity. Enzymes were incubated with DDT for 60 minutes and the products of metabolism monitored by HPLC. Results showed loss of the DDT substrate peak with concomitant production of a DDE peak (Fig 4.11). This confirms DDT dehydrochlorinase activity in AsGSTe2.1 and AsGSTe2.2.

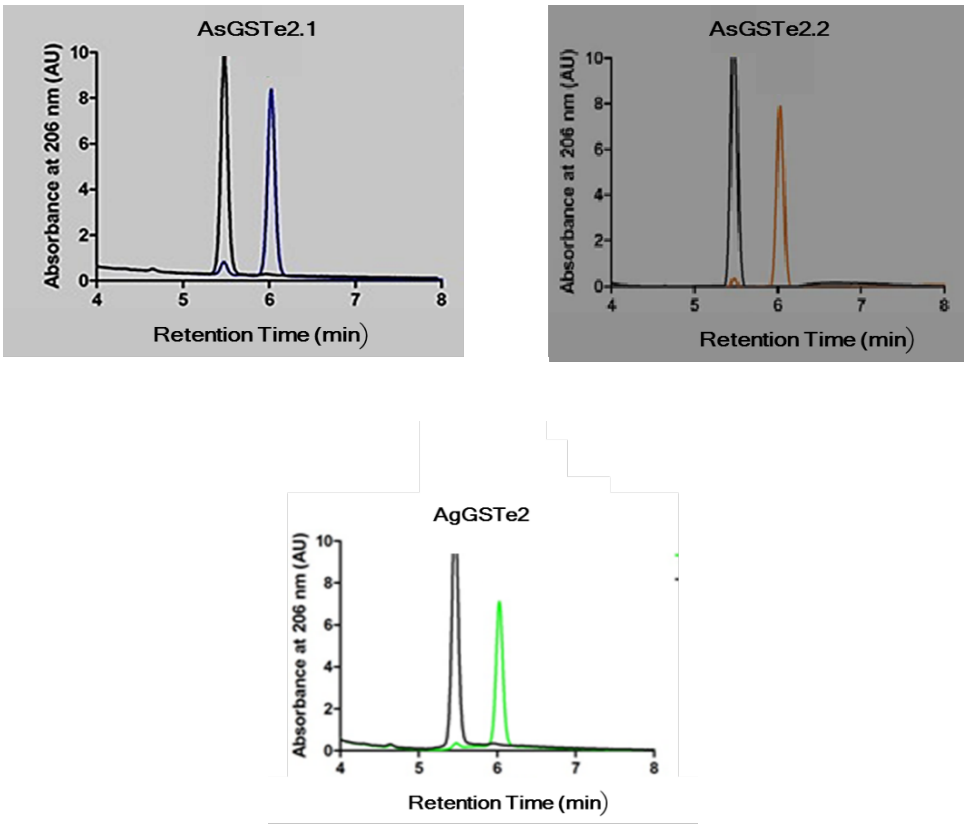


Figure 4.11 . DDTase assay illustrating the metabolic activities of GSTe2 variants, AsGSTe2.1, AsGSTe2.2 and AgGSTe2; with retention times of DDT and DDE were observed at 5.4 and 6.08 minutes respectively.

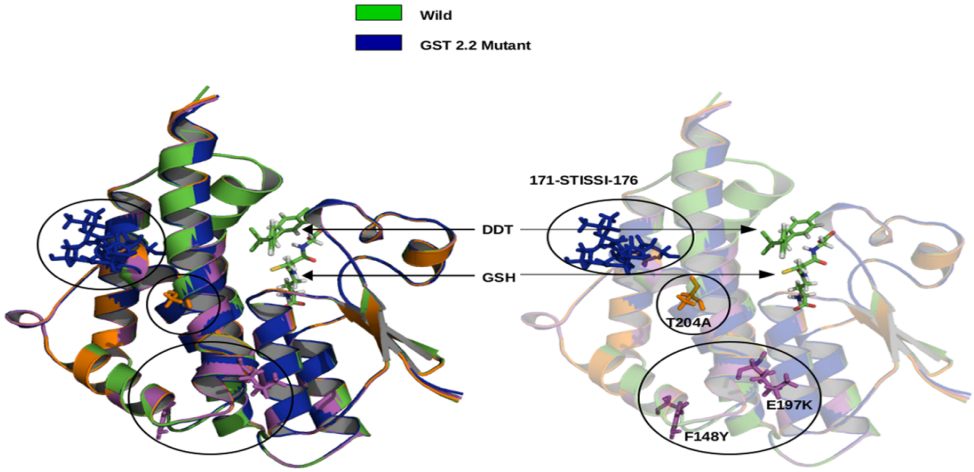


Figure 4.12. Mapping of polymorphic residues onto the transcripts structures AsGSTe2.1 and AsGSTe2.2 (exhibiting polymorphism). Mutations are marked within circles.

4.3.8 ASGSTe2 modelling and docking

The GST epsilon 2 protein variant sequences of *An. stephensi* derived and confirmed from cloning and sequencing were used for molecular modelling and docking with DDT ligand to gain perspective on the structural context of the variant residues in relation to the active site. Modelling results (Figure 4.12) show that all the polymorphic residues lie away from the DDT putative binding site suggesting that these residues are unlikely to have any effect on the enzyme activity and DDT binding, , which findings are consistent with our experimental data. Docking was completed using Autodock Vina for all docking simulations. Autodock Vina scores the docked poses on the basis of calculated binding affinity. The selected AsGSTe2.1, which we considered as wild in our study show a higher affinity for the docked DDT molecule compared to the AsGSTe2.2 transcript. Having these models will aid future characterization of AsGSTe2 and its variants that might be found in the population.

4.4 Discussion

Increased levels of expression of two epsilon genes AsGSTe2 and AsGSTe4 were evident from qPCR profiling of epsilon genes in laboratory DDT strains of *An. stephensi.* Further characterization of these two genes for evidence of association with DDT resistance revealed synonymous polymorphisms with at least four variants of AsGSTe2 enzymes (AsGSTe2.1 – 2.4) and three variants of AsGSTe4 (AsGSTe4.1 – 4.3). Both recombinants, AsGSTe2.1 and AsGSTe2.2 showed DDT dehydrochlorinase

activity. Detailed kinetic measurements were not carried out, however AsGSTe2.1, which resembles the putative enzyme produced slightly higher DDT turnover under the experimental conditions used, with 85% DDT converted to DDE per hour compared with AsGSTe2.2 at 75%. These values were also within the range observed for *An. gambiae* (79%) and *Ae. aegypt*i (89%) (Figure 4.11).The data suggest that both the AsGSTe2 recombinants are functionally active against DDT and capable of detoxification. GSTe4 has been characterised and identified in various insecticide resistant mosquito populations. Field studies at LSTM identified GSTe4 variant as a possible contributor to the pyrethroid resistance in *An. gambiae* where its overexpression has been regularly associated with pyrethroid resistance (Wilding *et al*., 2015; Abdalla *et al*., 2014) while also reported to have expressed during temophos selection in Aedes (Rodriguez *et al*., 2013). AsGSTe4 is found significantly expressed in all the resistant laboratory strains where three AsGSTe4 variants demonstrated GST activity with the functional analysis showing that all three variants were capable of catalyzing conjugation of CDNB and DCNB to glutathione, reported in silkworm GST (Chen *et al*., 2015) thus providing increasing evidence for its role in insecticide resistance while no GSH dependent peroxidase activity to CHP was detected. Metabolic assays however showed that AsGSTe4, unlike AsGSTe2 does not directly metabolize DDT, which is consistent with other reports where GSTe4 has failed to show DDT dechlorinase activity (Ortelli *et al*., 2003). Therefore it may be playing another role either in secondary metabolism (glutathione conjugation and excretion) or possibly as an antioxidant agent in conferring pyrethroid resistance (Vontas *et al*., 2001) however its exact role remains to be investigated.

Recently, five of the eight GST epsilons-- GSTe1, GSTe2, GSTe3, GSTe4 and GSTe7 were described to have been found overexpressed in a DDT resistant Zan/U strain of *An. gambiae,* where GSTe2 and GSTe7 showed the highest expression however only GSTe2 metabolised DDT (Ding *et al*., 2003). A question if recombinant homodimers of the epsilon may be an advantage towards metabolizing DDT is explained by previous research where it showed that neither GSTe1 or GSTe4 homodimers possess DDT dehydrochlorinase activity (Ortelli *et al*., 2003).

Riveron's recent finding on GSTe2 changed the present thought on DDT metabolic resistance to be associated with the single haplotype CYP6G1 described in *D.*

melanogaster, the only known mechanism. However, such point mutations associated with metabolic resistance are known in other genes such as the esterase in housefly (Campbell *et al*., 1998) and the carboxylase in sheep blowfly (Claudianos *et al*., 1999).

Gene sequencing of AsGSTe2 shares 92.3% identity with *An. funestus,* 89.6% identity with *An. gambiae* and 70.5% identity with *Ae. aegypti*. Cloning and sequencing of AsGSTe2 showed duplication of 6 amino acids (Ser-Tyr-Iso-Ser-Ser-Leu) at amino acid position 172 with residues Met-Gly lying between the two repeats. In AsGSTe4, the substitution of amino acids at amino acid positions 3 (Arginine to Lysine), 89 (Serine to Arginine) and 213 (Leucine to arginine) is found. The presence of the variants in AsGSTe2 is recorded in field populations as well. More detailed enzyme characterization carried against model substrates for GST, and assessment of kinetic parameters of the recombinants using CDNB and DCNB showed enzyme activities of the two AsGSTe2 variants for CDNB showed AsGSTe2.1 having a 2.4 fold higher catalytic efficiency than AsGSTe2.2 (k_{cat}/K_m ratio at 67 mM^{-1} s^{-1} versus 28 mM^{-1} s^{-1}). Studies on *Ae. aegypti* (Lumjuan *et al*., 2011) also described two GSTe2 variants showing similar specific activities against model substrates and where DDT dehydrochlorinase activity between them did not vary significantly. However the resistant PMD variant had lower Km for DDT and Vmax (DDE formation/min/mg) compared to the susceptible.

Further, comparison of thermostability of the enzymes carried out revealed that the AsGSTe2 variants were much less thermostable than AsGSTe4. It is unclear if lack of thermostability in AsGSTe2 is due to protein sequence alteration. Recent work on protein thermostability using what is called a consensus approach demonstrated that relatively small changes in a protein sequence can result in large changes in the thermostability of the protein (DiTursi *et al.*, 2006). Work on the GSTe2 of *Aedes aegypti*, suggested that the presence of redundant cysteine compared to *An. funestus* and *An. gambiae* which have only one conserved cysteine residue could be a possible explanation for its comparably low thermal stability in the group (LSTM, ECG group). In *An. stephensi,* no such redundant residues were recorded in the protein sequence of the GSTe2. A basic explanation for such low thermal stability could be due to unforeseen experimental issues relating to protein extraction and purification which might require more optimization.

Crystal structures of GSTe2 have been produced from *An. gambiae, An. funestus* and *Ae. aegypti.* These provided us with a template for the production of structural models of the AsGSTe2 variants. The models however predicted the variant residues to lie outside the putative active –site for DDT binding. Docking simulations attempted in the study suggest that AsGSTe2.1 has a larger cavity to accommodate the DDT molecule compared to mutants.

Mutations L119F (Riveron *et al*., 2014), I114T and F120L (Mitchell *et al*., 2014) reported in the GSTe2 gene known to play a role in DDT metabolic resistance showed associations with permethrin and deltamethrin resistance (Riveron *et al*., 2014). However the mechanism is unclear as these insecticides are not metabolised (Lumjuan *et al*., 2010). It can be noted that these mutations were not observed in our *An.stephensi* laboratory colony or field samples screened thus far. Known polymorphisms identified so far in this species are from Sistan and Baluchistan with nucleotide variations at positions 21, 90, 105 and 198 (Djadid *et al*., 2008), a few , which now add to positions 149, 172, 177, 202 and 209 identified in this species from our laboratory strains and field collection. Correlation between the different allelic variants at the gene level with phenotypic resistance was not investigated in this study although laboratory findings revealed the resistant strain to have the AsGSTe2.1 and AsGSTe2.2 in heterozygous state. With AsGSTe2 and AsGSTe4 together showing overexpression in our DDT and DEL colonies, the fact that the sequential arrangement of these two genes, their over expression may possibly be due to a cis acting factor acting as a coordinated regulation or just another event of strong positive selection acting on the AsGSTe4 gene (Wilding *et al*., 2015). While it is interesting to study the role of such mutations and their association with resistance, the mutations identified in our *An.stephensi* samples did not enhance DDT dehydrochlorinase activity showing no correlation with DDT resistance

4.5 Conclusion

The current study describes the plausible role of AsGSTe2 in DDT resistance. AsGSTe4 variants showed GST activity but did not metabolise the insecticide DDT. AsGSTe2 variants identified metabolized DDT at similar rates. The study is the first to characterise allelic variants in GSTe2 and GSTe4 of *An. stephensi*, and to examine the protein conformation of the two AsGSTe2 variants.

Chapter 5. MOLECULAR CHARACTERISATION OF VOLTAGE GATED SODIUM CHANNEL IN *ANOPHELES STEPHENSI*

Abstract

Voltage gated insect sodium channels are essential for signaling in neurons and electrically excitable cells, and have receptor- binding sites for neuro-toxicants. Knockdown resistance mutations (*kdr*) in the sodium channel are known to confer resistance against DDT and pyrethroids in insects. Post- transcriptional modifications such as alternative splicing and RNA editing have been argued to contribute to the channel's structural and functional diversity by giving rise to isoforms bearing unique functional and pharmacological properties. In this study, we examined the sodium channel of *Anopheles stephensi* for mutations and post-transcriptional events to understand their association with DDT resistance. We identified classic *kdr* mutations L1014F and L1014S along with two new mutations Q695R and E1235G and synonymous mutations F968 and I987 in *An*. *stephensi*. Post-transcriptional modification examination showed alternative splicing phenomena to occur in the vector. Optional exons (2, 5, 12, 13 and 12+13), mutually exclusive exons (20 and 27), alternative acceptor sites (exons 18, 23a and b, and 24), larger exons fragments skipped (12-13, 23-27, 5-19, 1-21) and an intron retention between exons 15 and 16 were identified. Expression profile of splice variants in adults showed no association with resistance, with the structure and most of the splice patterns exhibiting similarity with other insect VGSC orthologues. RNA editing events at the *kdr*-locus were absent.

5.1 Introduction

Voltage gated sodium channels (VGSC) are intrinsic transmembrane proteins present in most excitable cells, where they initiate and propagate the rising phase of action potential in these cells by their rapid opening (Mason, 2011). These serve as primary targets for DDT and pyrethroids. The effects mediated by these two insecticide groups are by inhibiting inactivation and deactivation of the channel, which results in depolarization of the membrane. This subsequently leads to paralysis and death of the insect (Vijverberg *et al.,* 1982; Lund AE & Narahashi, 1983; Bloomquist, 1996). One of the mechanisms of insecticide resistance known is target site insensitivity, which

occurs through amino acid changes brought about by single nucleotide polymorphisms within the sodium channel. Such mechanisms underlying DDT and pyrethroid resistance in the arthropod phyla and insect world are well documented and known as knock down resistance (*kdr*) mutations. These mutations reportedly confer a resistant phenotype by altering the gating kinetics (Vais *et al.,* 2000; Oliveira *et al*., 2013) or by bringing about a reduction in insecticide binding (O'Reilly *et al*., 2006;Usherwood *et al*., 2007).

Figure 5.1 illustrates the general concept of the sodium channel structure and function. The channel is comprised of an α subunit, which is the principal subunit for a functional channel and β which is involved with channel gating modulation and cell-cell interaction, where it modulates multiple aspects (Sivakumar *et al*., 2015) such as channel gating and expression at the plasma membrane of the Na channel and cell adhesion (Isom *et al*., 2001). Both α and β sub units have distinct tissue specificity expression. An auxiliary-regulatory β subunit, the tipE protein has been identified in the insect VGSC. It is an acidic membrane protein of 50 kDa having two putative membrane-spanning domains, and known to stimulate functional expression of *para* VGSCs (Feng *et al*., 1995). The first insect VGSC gene, *para*, cloned from *Drosophila melanogaster* (Warmke *et al*., 1997) showed the primary structure of insect VGSCs to be similar to that of the mammalian VGSC.

The α subunit with a modular architecture consists of four internally homologous domains (labelled I—IV), each containing six transmembrane segments. The four domains fold together to create a central pore (Figure 5.2), by the coming together of the S5—S6 linkers or P segments of each domain (Yellen, *et al*.,1991), whose structural constituents determine the selectivity and conductance properties of the channel (Marban *et al*., 1998). The III and IV domain has a prominent role in Na ion selectivity as recorded from previous research on human sodium channel. Domain III, in which a lysine (K1237 in the I1 sequence) is critical for discrimination of Na ion over Ca ions (Heinemann *et al*., 1992; Chiamvimonvat *et al*., 1996; Perez-Garcia *et al*., 1997) and IV, in which mutations of various contiguous residues (1531—1534) render the channel non-selective among monovalent cations (Chiamvimonvat *et al*., 1996). The general strategy for activation gating is highly conserved: the fourth transmembrane segment (S4), stereotypically studded with positively charged

residues, lies within the membrane field and moves in response to depolarization, somehow opening the channel (Stuhmer *et al*., 1989).

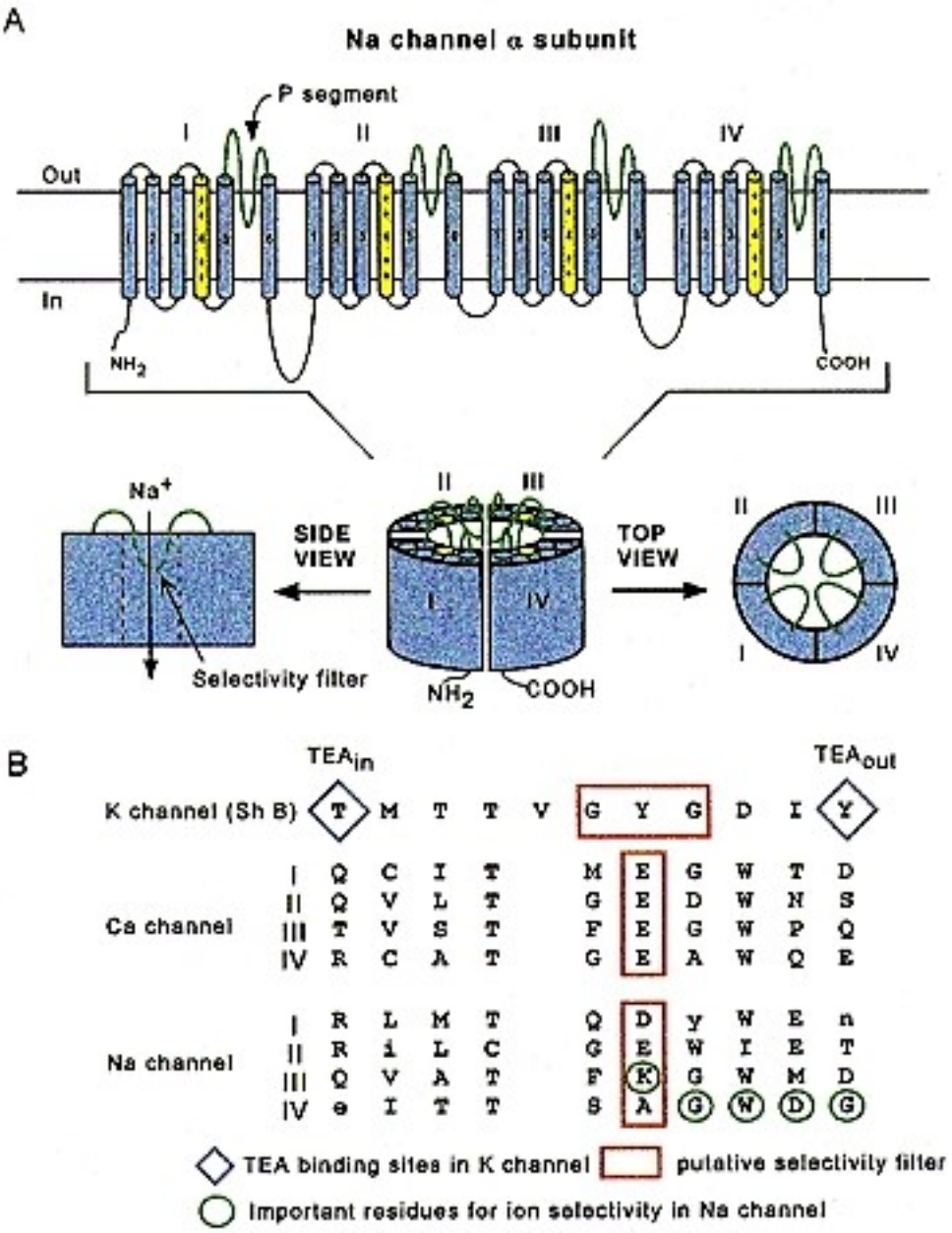


Figure 5.1. Schematic depiction of the alpha subunit of the Na^+ channel (adapted from Marban *et al*., 1998). **A**- Illustrates the putative transmembrane folding where yellow shade illustrates the S4 segment and green for pore lining P segments. **B**- aligned primary amino acid sequences in single-letter code of the P segments in a K^+ channel (*Shaker* B), the four domains of the cardiac L-type Ca^{2+} channel, and the four domains of the Na^+ channel. Upper cased residues are highly conserved among voltage-dependent Na^+ channels. Red boxes outline the putative selectivity filters; in case of the Na^+ channel, the residues of most importance for selectivity (circled in green) are mostly outside the box.

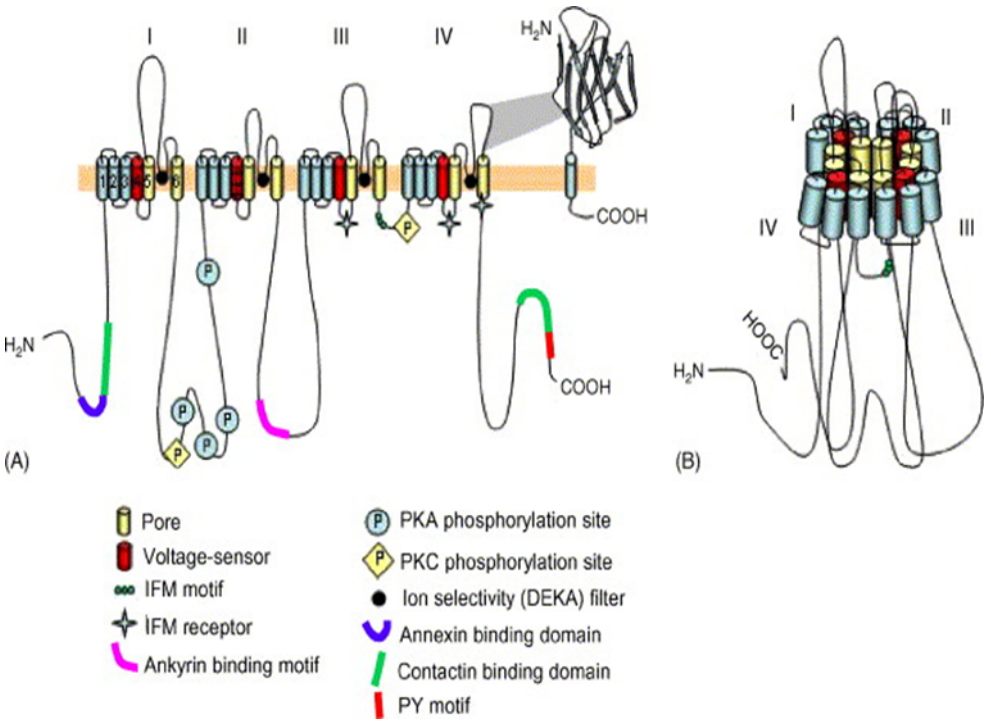


Figure 5.2. Topology of the voltage gated sodium channel. A: 2D representation of a subunit with the b subunit showing domains and the transmembrane helixes. Different representations of colours and shapes for different functional sites. B: the α unit folding to form the Na+ conducting pore (Adapted from Ekberg *et al*., 2006).

Much research on the VGSC has focused on human therapeutic drug effects. Evolution of insecticide resistance in the recent decades has drawn attention on the role *kdr* mutations play in the VGSC. Resistance against the knock down effect of DDT and pyrethroids insecticides whose primary target resides in the VGSC is studied in most insect species. Studies confirmed the role of *kdr* mutations L1014F (Martinez *et al*., 1998) and L1014S (Ranson *et al*., 2000) besides other *kdr*-like mutations in a series of insects in the arthropod phyla (Silva *et al*., 2014) with pyrethroid resistant phenotype. Whilst *kdr* mutations are widespread and associated with DDT and pyrethroid resistance, posttranscriptional events such as RNA editing and alternative splicing were recently hypothesized as modifying the gene at the protein level to achieve sodium channel diversity with altered gating kinetics in a few insects such as the German cockroach (Song *et al*., 2004; Tan *et al*., 2002; Liu *et al*., 2001), and more recently reported in a psocopteran fly (Jiang et al., 2013). Others however argued that such events were absent (Chang *et al*., 2009; Zhifeng *et al.,* 2013). RNA editing in Drosophila para sodium gene is functionally inferred to affect phosphorylation, thus prolonging the slow inactivation in the channel (Reenan *et al*., 2000). A novel voltage

gated cation channel with a close structural and evolutionary relationship to voltage-gated Na^+ but with many properties of Ca^{2+} channels was functionally identified in German cockroach, although the mechanism is not understood (Zhou *et al*., 2004).

In this study, to gain a clearer understanding of the VGSC and its role in insecticide resistance, the gene sequence of VGSC of *An.stephensi* DDT resistant laboratory strain was characterized. The aim of this study was to examine the *kdr* gene for *kdr* like mutations apart from the classic mutations already reported in this vector (Singh *et al*., 2011), and examine post transcriptional modifications such as RNA editing and alternative splicing in the VGSC transcript to determine relationship with insecticide resistance.

5.2 Material and methods

5.2.1 Mosquito maintenance

Rearing of *An. stephensi* in the laboratory is as described in earlier chapters with insectary conditions as mentioned. Lines were derived with the help of bioassays and each line was segregated into separate cages. Eggs, third instar, fourth instar, pupae and adults intended for experiments were collected and stored in RNA later at -80°C until use.

5.2.2 DNA and RNA isolation

Co extraction of total RNA and DNA was carried out from RNAlater preserved specimens using an RNeasy micro kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol but incorporating gDNA elimination step using DNAse 1 kit (Promega) prior to final elution of the RNA. The purity and concentration of the eluted RNA was checked on a Nanodrop (Thermofisher) measured at 260nm and 280nm. 1% agarose gel electrophoresis was used to run the freshly extracted RNA to check the integrity.

5.2.3 Complementary DNA synthesis (RT-PCR)

About 1ug of total RNA was reversed transcribed using Maxima hot-start first strand cDNA synthesis kit (Fermentas, ThermoFisher Scientific, USA) using oligo (dT)18 following the manufacturer's instructions although with slight modification on the incubation step i.e., keeping 42^0C as our standard incubation temperature for obtaining a full length (6.5kb) cDNA . Synthesized cDNA was aliquoted for single thaw and use and stored at -20°C until use.

5.2.4 PCR amplification

For amplification of the VGSC gene, sequences from closer species were aligned and the sequence with the highest similarity was extracted from VectorBase. Primers were designed from the untranslated region (UTR) of the gene (Figure 5.3). First amplification product was sent for sequencing to validate the sequence and final primers were designed from the sequenced product. The primers UF and UR (Table 5.1) were flanking primers used for amplification, and internal primers were used to amplify in case of amplification failure. The PCR amplification was carried out using Phusion high fidelity Taq (New England Biolabs, Ipswich, MA) as a polymerase and the PCR conditions were an initial denaturation at 98ºC for 30 seconds followed by a denaturation at 98ºC for 10 secs, an annealing at 60ºC for 30 seconds and an extension at 68º for 5 minutes for 35 cycles of each, with a final extension at 68ºC for 7 minutes.

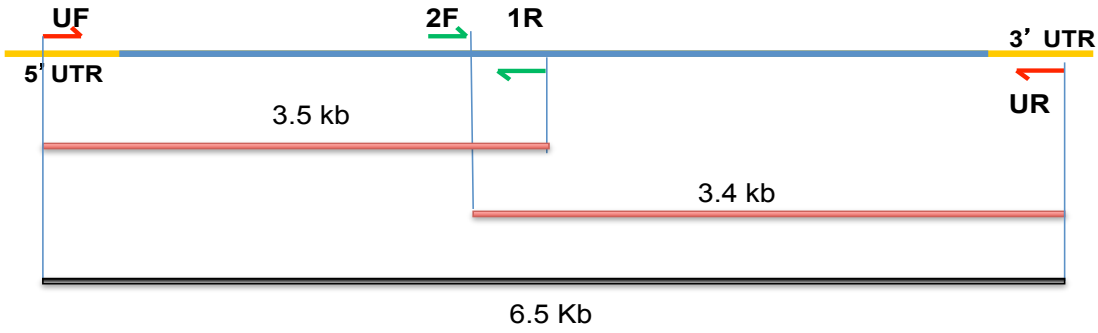


Figure 5.3. PCR amplification strategy for the 6.5 kb voltage gated sodium channel gene in *An. stephensi.*

5.2.5 Cloning and sequencing

PCR amplified products of the VGSC of *An. stephensi s.s* were cloned in cloneJET vector using the cloneJET PCR cloning kit (Thermo Fisher Scientific) intended for blunt ends PCR generated products. Clones were PCR re-amplified and purified using Qiagen PCR purification kit and the purified products were sent to Macrogen Inc, South korea for sequencing.

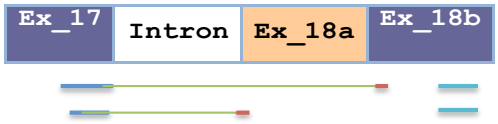
Table.5.1. List of primers used for general amplification, genotyping, sequencing and cloning in the study.

	Amplification, Cloning and sequencing
UF	CCT TGG ACA TCA GAG GCT CT
UR	ATT ATT TCT GCC GTG CTT CG
1R	CGA TGA AGA ACC GAA ATT CGA CAA AAG CAA GGC T
2F	GGG ACT GTA TGC TTG TCG GTG ACG TG
C4F	CTT TGC TAT CAT GGG GGT GCA
C4R	CGT TCG CGA TGT TAT GCT GGG
C3F	GTG ATG GGA ATG CAG CTG TTC
C3R	TGC ACC CCC ATG ATA GCA AAG
C1F	ATG ACC GAA GAC TCC GAT TCG
C1R	TAC GTA TCG TGA ACT GCA GTC GGT
5UTR.1F	CCT TGG ACA TCA GAG GCT CTC ATT GTT GGC
3UTR.1R	TTA TTT CTG CCG TGC TTC GGA ATC TGA ATCC
EX21.1F	GGG ACT GTA TGC TTG TCG GTG ACG TG
EX33.1R	CGA TGA AGA ACC GAA ATT CGA CAA AAG CAA GGC T
EX3.1F	CAG GGT GTA CCA GTC CCA GTT CGA AT
EX5.1F	CTA TTA CTA CGA AGT CCA ACC AAT TCC ATG CA
EX11.1R	CTT TAC GGG CTT TTG CAG TGC CAG C
SP6	ATT TAG GTG ACA CTA TAG AA
T7	TAA TAC GAC TCA CTA TAG GG
	Genotyping
KdrF	GGA CCA YGA TTT GCC AAG ATG
kdrR	CGA AAT TGG ACA AAA GCA AAG
StF	GAT TGT GTT CCG TGT GCT GT
St_leu	GCG GGC AGG GCG GCG GGG GCG GGG CCC GAT CGG AAA GTA AGT TAC TTA CGA gTA

St-Ser	CGA TCG GAA AGT AAG TTA CTT ACG AtT G
St_LS1	GCG GGC AGG GCG GCG GGG GCG GGG CCC GAT CGG AAA GTA AGT TAC TTA CGt CT
Stphe	GAT CGG AAA GTA AGT TAC TTA CGg CA
1235R	CAT CTG ATT TTT GCA GGA CAT GAC CCG
Q1235F	GAC TGA CTG ACT GAC TGA CTG ACT GAC TCA TCT GAT TTT TGC AGG ACA TGA CCC A
R1235F	GGT TTG GGT TTG AGC AGG TTC TAT GAA AGG3'
R695F	TCT TCC ACA GAA CTC GAC GAC GAA GGA GG
Q695R	ACC ATC GAG CGG ACC TTC CTC GCA CT
695F	AAA TCG CCC ATT CAA GGA CGA AAG CCA C
695R	TCC GGC GAG AAC CGG GAA TTT TTT GTA G

5.2.6 Quantitative PCR and RNA sequencing

Relative expression of the splice variants were examined using qPCR. Primers pairs used in the qPCR are provided in Table 5.2. Primer designing strategy employed in this study is illustrated in Figure 5.4 where intron –exon overlaps were considered.



Ex18S ACAGTAGAACTAGTGATCACGGAG**AG**
Ex18L CGGAG**TCTCTGTTTATTACTTCCCC**
Ex18R ACAACAGTCCCAGACGCAGAAA

Figure 5.4. Primer designing strategy used for splice isoforms expression for partial exon 18.

Table.5.2. List of primers designed for qPCR of splice variants in VGSC.

Primer	Sequence 5'-3'
Exon2ssF	AGC GAG CCG AGG GGG ATA
Exon2ssR	TGC ATT CGA ACT GGG ACT GGT
Exon2llF	AGC CGA GGG GGA GTG ATT TT
Exon2llR	TGC ATT CGA ACT GGG ACT GGT
Exon5ssF	GCC GAC AGT CGA ATC TAC CGA ATA T
Exon5ssR	TGA ATG TTC TCA ACG CAG CGA G
Exon5llF	AAA GTG ATG GCG CGA GGT TT
Exon5llR	AAC GCA GCG AGA TTA CCC AGA T

Exon11ssF	TTG GCC ATT GTT GCC ATG TCG
Exon11ssR	TGA ACA GTA GCG ACC CCC C
Exon11llF	TTG GCC ATT GTT GCC ATG TCG
Exon11llR	TCC GAT GGG CTT TTT GCG AT
Exon12ssF	AAC AAC CGC ACC AAC AGC TA
Exon12ssR	CCG GGT AAT GAA AGT GAA GCC G
Exon12llF	GGG GGT CGC TAC TGT TCA AAA
Exon12llR	TGA TGT GAT CCT CTA GAT CCT CTA CG
Exon13ssF	AAC AAC CGC ACC AAC AGC TA
Exon13ssR	TGA ACA GTA GCG ACC CCC G
Exon13llF	GGC TTC ACT TTC ATT ACC CGG TTC
Exon13llR	ATC GGC GTA CGG CAA ATG TT
Exon18S	ACA GTA GAA CTA GTG ATC ACG GAG AG3'
Exon18R	TCC ACG AAT GGG TCG AAC ACA A
Exon18L	CGG AGT CTC TGT TTA TTA CTT CCC C
Ex20cF	GCA AAG TCC TGG CCT ACA TT
Ex20cR	ACG AAG GTC AAA TTG CCC AA
Ex20dF	ATG GGC AGA ACG ATG GGA G
Ex20dR	CGA ACA GCT GCA TTC CCA TC
Exon23_63bsF	GCA TCC GTA CAA CCG ACA GAT
Exon23_63bsR	CAG CTG GTT GTG CTC CTT GAT T
Exon23_63bLF	ACA ACC GAC AGG CAA AGG AGT
Exon23_63bLR	AGC TGG TTG TGC TCC TTG ATT C
Exon24ssF	GCG ATG GCA TGG AAT TCA CCA
Exon24ssR	GAT ACT GGC AGT ATC ATC GTC CTT T
Exon24llF	AAT CAT AGA GGC GTG TCC TTG C
Exon24IIR	TGC TGG CGT CAC GTT TTT CT
Ex27lF	TTC GTT GCT TCA CTT TGT GG
Ex27lR	CCC TGC ATA CGG GAC AT
Ex27kF	TAA ACC TTG CCG CTA TCT GG
Ex27kR	CAT ACC CTC CCA GCG TGA TA
Actin_F1	TCC GTG ACA TCA AGG AGA AG
Actin_R1	TTA CCG ATG GTG ATG ACC TG

Taq_S7F	TGC GTG AAT TGG AGA AGA AG
Taq_S7R	GGT CTC TTC TGC TTG TTG GG

5.3 Results

5.3.1 Full coding sequence of VGSC

The full coding sequence of VGSC *An.stephensi* was successfully amplified. Characterization of the DNA sequence following amplification and cloning (in two overlapping fragments) on aligning using online software Multiple Sequence Comparison by Log- Expectation (MUSCLE) showed it to be composed of 6424 nucleotides with an average length of 2141 amino acids (MUSCLE v3.8.31). Two new mutations Q695R and E1235G were found in co-occurrence with the L1014 residue of the susceptible *An. stephensi.* Classic *kdr* mutations L1014F and L1014S were also present. The roles of the two novel mutations were not examined since they were absent in field population examined and were recorded in only one batch of the susceptible strain. Synonymous mutations were also observed. The full coding sequence of the VGSC in *An. stephensi* is given in Appendix 5. A distinctive feature is the occurrence of a repeat of nucleotides in the 5'end of the UTR region, which is seen in a few clones. This nucleotide repeat region consist of quadruplet repeats of thirty nucleotides in the sequence 5'UTR (CCTTGGACATCAGAGGCTCTCATTGTTGGC) -3'. Whether such duplication of nucleotides is at the gDNA is yet to be ascertained.

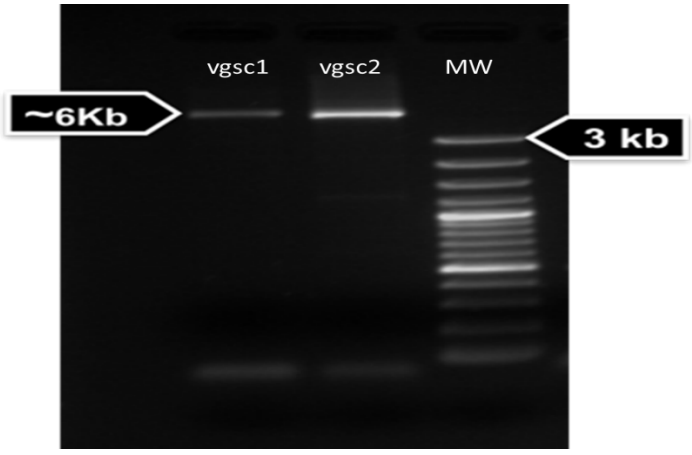


Figure 5.5. PCR amplified 6 kb product of the coding VGSC of laboratory *An. stephensi,* MW= 1kb ladder, vgsc1 and 2 = 6 kb products of DDT/ pyrethroid resistant and a susceptible strain respectively.

5.3.2 RNA editing

The *An.stephensi* strains with homozygous L1014, 1014F and 1014S alleles were examined for RNA editing events at the *kdr* locus 1014. Unlike reports in other insects, (Liu *et al*., 2004; Song *et al*., 2004), the VGSC of *An.stephensi* revealed that RNA editing phenomenon is absent at the classic *kdr* site, 1014 locus. The conclusion was based on the fact that there was no difference in the deduced amino acid sequence in cDNA compared to the VectorBase sequence. Sequencing of gDNA and cDNA for IIS6 trans-membrane segment of VGSC for mosquitoes with L1014, 1014F and 1014S alleles did not show any changes at the allelic level in both the RNA and DNA, suggesting no editing phenomenon. RNA editing anomalies, however, were observed in our direct sequencing experiments and are illustrated in Figure 5.6 where a G peak is found to occur in all samples examined for the residue. To clarify if such events are real, we cloned the PCR product of each corresponding sample and sequenced the clones. We confirmed that the events are sequencing artifacts and that no editing is seen at the 1014 locus in this vector.

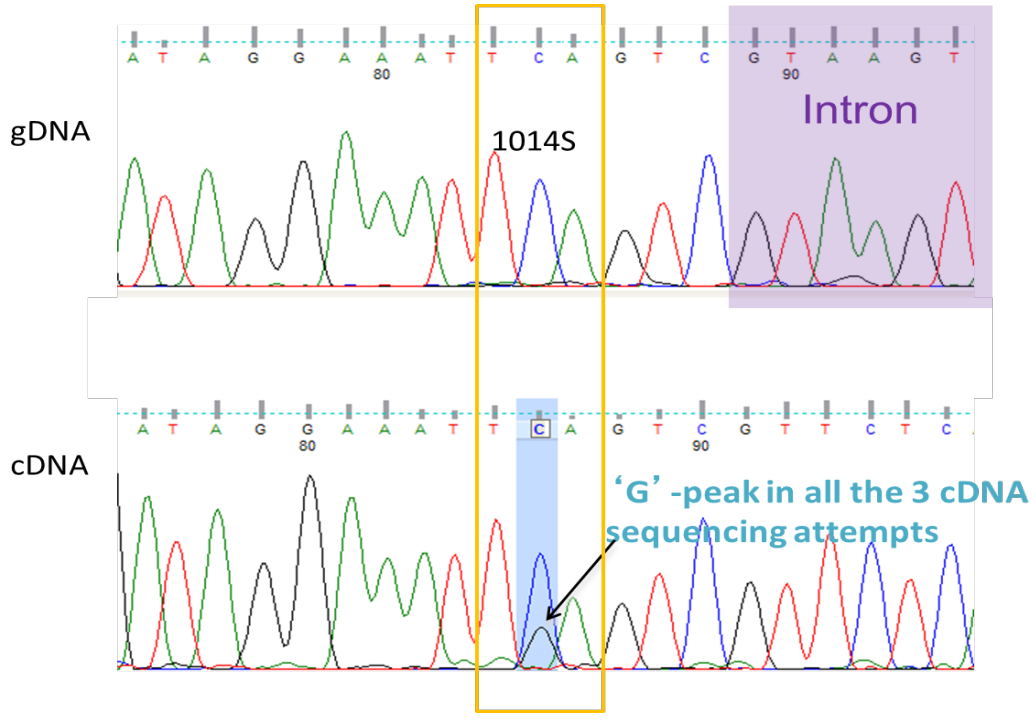


Figure 5.6. G-peak arising from sequencing artifact proven through cloning that RNA editing does not exist at the *kdr* locus.

5.3.3 Alternative splicing and expression of splice variants

Cloning and sequencing of the whole coding region of the VGSC was successful by primer walking with different primer combinations. The analysis revealed extensive splicing in the VGSC of the Indian *An.stephensi*. To validate alternative splicing events recorded in our *An. stephensi* sodium channel sequence analysis, different exon-overlapping primers were employed for amplification and for expression profiling of the splice variants using quantitative PCR. This was done to avoid any form of contamination, which is unseen during RNA isolation which may consequently lead to amplification of any intronic portion lying between the spliced exons. Evidence of alternative splicing is recorded in this urban vector in the study.

Sixteen alternative splicing events recorded in the VGSC of *An.stephensi* include nine exon skipping, two mutually exclusive exons i.e., exons 20 and 27, four alternative acceptor sites i.e., exons 18, 23, 24 and an intron retention between exons 15 and 16 (Table 5.3). It was observed from cloning experiments that optional exon 2 and partial splice variant of exon 18 was predominant in pyrethroid strains. Such difference was not observed in field population. Alternative splice events such as skipping of exon 2, exon 5, partial splicing in exons 18 and 24, and the four mutually exclusive exons in 20 and 27 are found to be commonly reported in most insects including the African vector, *An. gambiae*. Large skipping of exons has been noted in three events, one of which has been observed in *Culex* species as well where exons 5-18 were found absent in the culex VGSC transcript, CxNa-S_v1 which also lacked exons 2, 5 to18, and 22 resulting in a short sodium channel sequence (Lin *et al*., 2012). Skipping of exon 5 to exon 19 is observed in this study in the resistant clones and is found linked to a skipped exon 2. We also observed exon 23 to exon 27 spliced out in *An. stephensi*, and skipping of exon 1 to exon 27 although present is limited to the susceptible strain only. Exon 23 of *An.stephensi* exhibited two different splice events along the same stretch where one splice variant showed a 63 base pair splicing and another with a 30 base pairs spliced out. The different alternative splicing events recorded in urban malaria vector *An. stephensi* are illustrated in Figure 5.8.

Expression of alternative splice variant(s) in DDT- and pyrethroid- resistant and susceptible *An. stephensi* was investigated. Quantitative PCR strategy was employed

to study the relative expression of splice isoforms in DDT and pyrethroid resistant and susceptible strains to validate cloning results. Splice variants belonging to exon 2, exon 5, exon 18, exon 23 and exon 24 of *An.stephensi* mosquitoes examined showed that there is no significant difference in the splice variants of the DDT/pyrethroid and the susceptible strains. Copy number variation determination was not attempted in the study.

The adult *An.stephensi* laboratory strains showed some differences in expression level of the exons with spliced exon 18 and skipped exon 2 having comparable expression levels. Spliced exon 18 in adult mosquito also showed higher expression to its counterpart where splicing event is absent. However, it is clear that no difference exist between the susceptible and the resistant strains as evident in Figure 5.7.

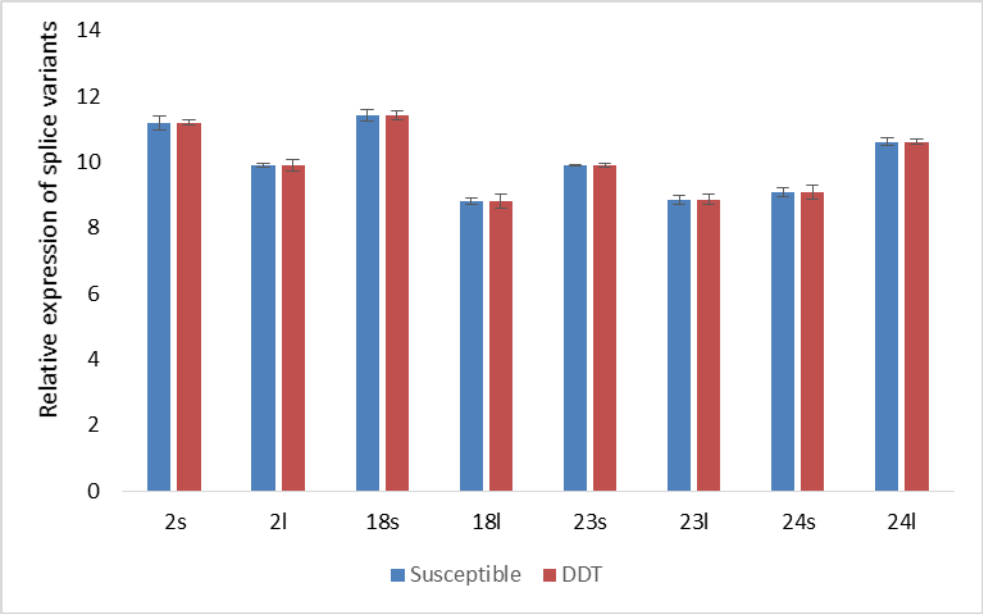


Figure 5.7. Relative expression of splice variants of exons 2, 18, 23 and 24 in adult *An.stephensi* and abbreviations SS- susceptible strain; RR – DDT Pyrethroid resistant strain; s- spliced; I – w/o splicing event. Error Bar represents the SD. i.e. p-value summary is non- significant (P <0.2) between susceptible and resistant strains.

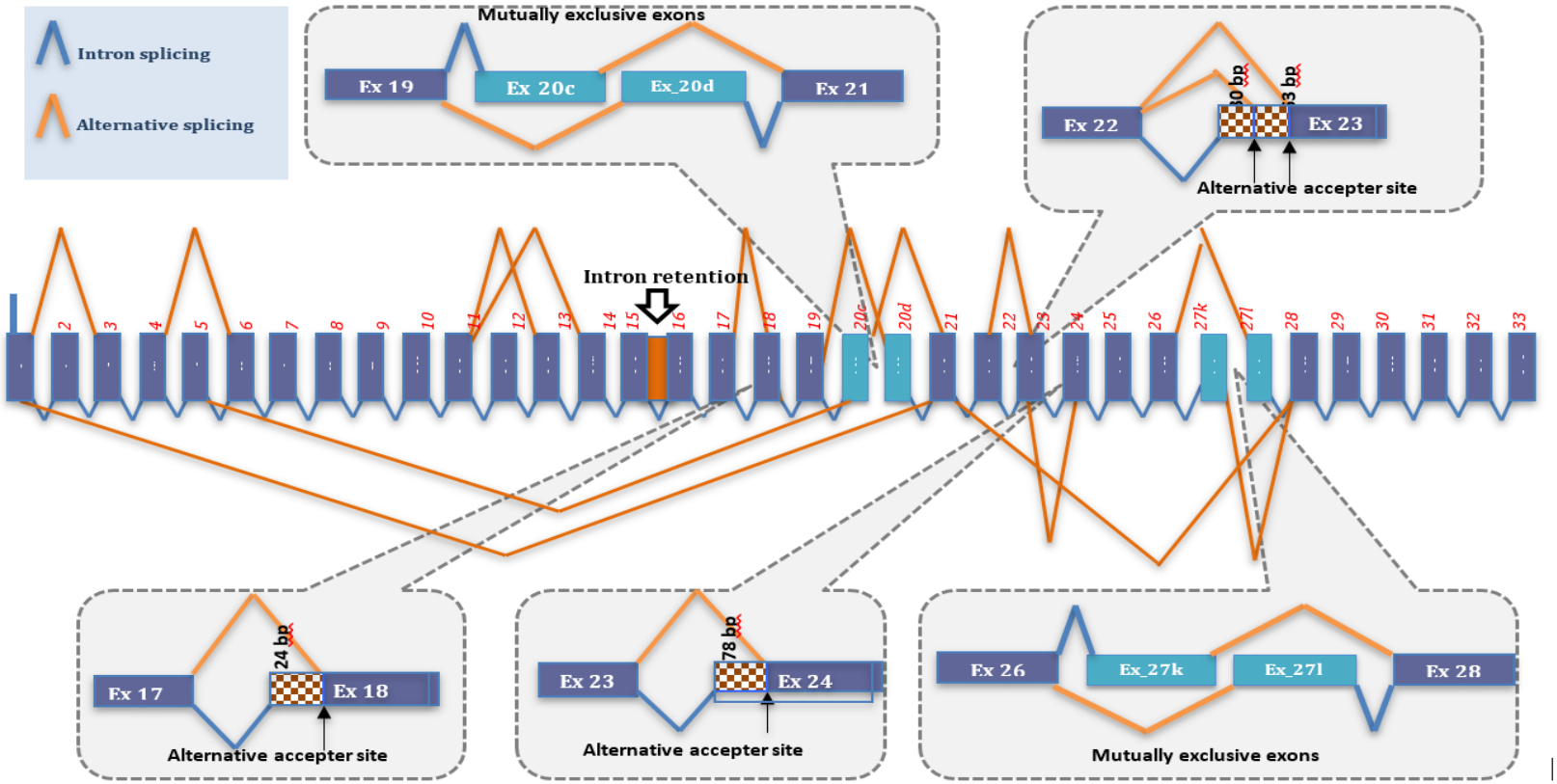


Figure 5.8. Diagrammatic representation illustrating the different alternative splicing events occurring in the VGSC of *An. stephensi*.

Table 5.3. Alternative splicing events evident from *An. stephensi* clones and those reported in other insect species.

Description	***Occurrence in R and S***	***Type***	***Reference***
Skipped Exon 2	> R/ < S	Reported in most insects & *gambiae*	Davies *et al.,2007* Lin *et al*., 2009
Skipped Exon 5	R	Reported in most insects & *gambiae*	Davies *et al*.,2007, Lin *et al*., 2009
Skipped Exon 11			
Skipped Exon 12			
Skipped Exon 13		*Drosophila*	Olson *et al.,* 2008
Skipped Ex 12 &13			
Spliced Exon 18 (24bp)	>R/ <S	Reported in *gambiae*	Tan *et al*., 2002 Davies *et al.,2007* Olson *et al.*, 2008
spliced Exon 23 (63bp)	R		
Spliced Exon 23 (30bp)	R		
Spliced Exon 24 (78bp)	>R/<S	Reported in *gambiae*	Davies *et al*.,2007
Skipped Exons 23-27	S		
Skipped Exon 5- 19 (occurs with skipped exon2 in *An.stephensi*)	R	Reported however 5-18 only in culex	He *et al* ., 2012
Skipped Exon 1- 21	S(1)		
Mutually excl 20 c/d	R showed >d <c	Reported in most insects	Davies *et al*.,2007 Jiang *et al*., 2013
Mutually excl 27 k/l		Reported in most insects	Tan *et al*., 2002
Intron retention between exon 15-16	S (2)		

Abbreviations: R- DDT-pyrethroid resistant; S- susceptible; excl-exclusive; (2) - in only two clones

5.4 Discussion

The voltage gated sodium channel is a primary target of DDT and pyrethroids in insects where modifications within the protein structure in particular, established point mutations led to resistance in the insect against the insecticide. The VGSC is well studied in eukaryotes particularly in humans where 9 channel pore forming α-subunits were identified (Ahern *et al*. 2016) with much work focused on drug interactions structure – function relationships (Duchlohier, 2009). *Kdr* mutations in the channel are reported to confer resistance against these insecticides in numerous insect species. Silva *et al*., 2014 reviewed the *kdr* mutations prevailing in the voltage-gated sodium channel gene of anophelines and their association with pyrethroid resistance. With much work already done in other species, the voltage gated sodium channel in *An.stephensi* has been characterized in this study. Our current observations are in line with our previous records (Singh *et al*., 2011) in the species which revealed that classic *kdr* mutations 1014F and 1014S were present. The 1014S mutation predominated over the 1014F allele in a field survey (Dykes *et al*., 2016) and is found to have a higher survivability under laboratory conditions. The presence of the two mutations Q695R and E1235G was observed in a few clones of one laboratory colony but is not observed in any of the field collections genotyped, leaving their role therefore unknown. The functional polymorphism of the classic *kdr* mutations L1014F/S in the sodium channel in insect pest and malaria vectors has long been associated with DDT and pyrethroid resistance (Ibrahim *et al.,* 2014; Martinez-Torres *et al.,* 1998; Ranson *et al.,* 2000*)*.Research findings assert that the relation between *kdr* genotype and DDT and pyrethroid susceptibility or resistance as observed in a wide range of taxa holds true (Donnelly *et al*., 2009) while other contend that such genotype- phenotype of *kdr* relation may not be true in all insect species.

Over the years, with resistance emerging against an array of insecticides used for most disease vectors, the molecular basis behind resistance became an essential area of focus. Later studies focusing on the RNA level questioned whether such polymorphism of the L-to-F/S *kdr* mutation at the genomic DNA level mirrors a true picture of the association with insecticide resistance. Investigations on the single nucleotide polymorphism for alleles at the *kdr* locus of mosquitoes which underwent DNA variation report that no strong correlation was found between *kdr* genotype and resistance phenotype. On the contrary, they corroborated critical evidence that the

presence of allelic variations at the RNA level of the *kdr* locus showed strong association with susceptibility levels and resistance of mosquito populations although not reported in anophelines. Recent studies however suggest that the sodium channel diversity may be achieved through post transcriptional modifications such as alternative splicing (Davies *et al*., 2007; Lin *et al*., 2009) and/or RNA editing (Du *et al*., 2006; Liu *et al*., 2004).

Post transcriptional modification, which include alternative splicing events and channel editing are suggested to have amplified the repertoire of voltage-gated sodium channels in some insect species. RNA editing as a ubiquitous phenomenon alters the information content in an RNA molecule following transcription from genomic templates. The molecular processes involved include nucleobase modifications such as a cytidine to uridine and adenosine to inosine deamination besides additions, and insertions that are non-templated. Findings limited to German cockroach (*Blattella germanica*) (Liu, Song, & Dong, 2004; Song, Liu, Tan, Nomura, & Dong, 2004), fruit fly, *Drosophila melanogaster* (Hanrahan *et al*., 2000) and mosquito *Culex quinquefasciatus* (Xu *et al*., 2011; Xu, Wang, Zhang & Liu, 2006) and *Ae. albopictus* report evidence of RNA editing in the voltage gated sodium channel protein. RNA editing events in the findings described that allelic variations happening at the RNA level might attribute to a phenotype or trait, for instance resistance to insecticides at the target site. A U-to-C editing identified in *Blattella germanica* sodium channel (BgNav) and Drosophila sodium channel (DmNav) showed persistent current (Liu et al., 2004; Song et al., 2004) while A-to-I in DmNav showed low voltage activated sodium channel protein (Olson *et al*., 2008).

Studies carried out on L-to-F *kdr* allelic variation at RNA level suggest that identification of the L-to-F *kdr* allelic variation at the genomic DNA level is questionable from such findings and that functional polymorphism of the *kdr* mutation in the sodium channel that was associated with insecticide resistance of mosquitoes was not a subject of the undergoing DNA variation since no significant change is seen in the genotype between susceptible and resistant strains or between the field collected resistant parental strain and its offspring that had undergone eight generations of selection in the laboratory with permethrin although their levels of resistance to permethrin are dramatically different (Xu *et al*., 2006). It may be that the

mechanism of gene recoding might involve imperfect base pairing of exonic and intronic sequences.

It has been described that such events are said to occur almost exclusively in gene products whose primary function affects neuronal signaling (Hoopengardner *et al*., 2003, with a number of human neurological diseases found associated with this re-coding system (Gurevich *et al*., 2002; Kawahara et al., 2004). A-to-I RNA editing was reported by Reenan and group to be the most common form of editing which occurs in the ion channels of most insects species and which vary between arthropod species much from their observation in the ideal *Drosophila* sodium channel (Reenan *et al*., 2000), where multiple genetic techniques are feasible in the organism (Gott, 2007). Ten A to- I editing sites were identified in the para transcript of *Drosophila* where 8 of them result in amino acid changes in the intracellular linkers, the actual number was said that it may be much greater than ten but none of these sites were observed in BgNav (Dong, 2007) apart from two A-to-I and three U-to-C editing sites with amino acid changes in the transmembrane segments. RNA editing events in the VGSC of BgNav and *Drosophila* put forward a strong support of the notion that channel kinetics and neuronal excitability is regulated by such mechanistically driven events or in other words, that sodium channel current diversity or neuronal excitability changes are achieved through RNA editing.

Our investigation of SNP for TTA i.e. TCA in the case of Serine and TTT for phenylalanine alleles of the *kdr* locus at the genomic DNA and RNA level in 30 individuals from each homogeneous *kdr* line showed no presence of the *kdr* allelic variation at the RNA level for all genotype combinations of the *kdr* in the sodium channel locus L1014 of *An. stephensi*. Since 30 mosquitoes in total were sequenced, a correlation with levels of susceptibility and resistance to insecticides is immature to conclude. It is essential however to note that, contamination from surrounding intronic region which Martinez-Torrez *et.al* defined, might be the cause behind findings which documented the role of allelic variations in relation to insecticide resistance. The idea was refuted confirming that no introns were detected in the region as against that reported by Martinez-Torres *et al*.

Another post transcriptional modification-- alternative splicing is suggested where splice variants are said to affect the VGSC channel kinetics in the insect species

studied. Alternative splicing events have been reported in *Drosophila* (Lin *et al*., 2009) cockroach (Tan *et al*., 2002), culex (He *et al*., 2012), booklouse (Jiang *et al*., 2013), while RNA editing is found in the sodium channel of *Drosophila* (Hanrahan et al., 2000; Olson *et al*., 2008; Reenan *et al*., 2000) and cockroach (Liu *et al*., 2004; Song *et al*., 2004). Sodium channel activity is described to possibly be modulated by alternative splicing where drastic differences in the channel expression and gating properties have been described to play a role in diversification of VGSC activities in vivo (Dong *et al.,* 2014). Editing sites (A-I) present in *Drosophila* are not found in the cockroach, however two different A-to-I RNA editing sites and three U-to-C editing sites are present in German cockroach, BgNav, each said to result in an amino acid change in the transmembrane segments (Liu *et al*., 2004; Song *et al*., 2004). The U-to-C editing site, F1919S, is found in DmNav (Liu *et al*., 2004). While functional alterations in the channel due to RNA editing is unknown in *Drosophila*, such events in BgNav transcripts are reported to cause subtle shifts in the voltage dependence of activation and/or inactivation (Song *et al*., 2004). Seeburg 2000 hypothesise that in vivo RNA editing events might have a hand in fine tuning neuronal activity is upheld by most protagonists of such events in insects.

Examination of alternative splicing events in the VGSC of *An. stephensi* in this study revealed sixteen splicing events. Skipped exons 2 and 5 and partially spliced junctions at exons 18 (24 base pairs spliced) and 24 (78 base pairs spliced) were previously reported in *An. gambiae* and in other insect species (Davies *et al*., 2007). Our cloning experiments showed that skipping of exon 2, exon 5 and exon 18 were predominant in the pyrethroid resistant strain. However this was not supported by qPCR expression analysis of the event variants suggesting these events may not have any role to play in resistance. Spliced exon 18 show a higher level of expression compared to the rest in adult *An.stephensi*. Mutually exclusive exons 20 c and d and exons 27 k and l recorded in this study were previously reported by Davies group in the African *An. gambiae.*

While a new optional exon 23 located in the linker between domains II and III, and four mutually exclusive exons (named as exons 27A, 27B, 27C, and 27D) in domains IIIS3 and IIIS4 of the sodium channel of *Liposcelis bostrychophila)* were reported, in this study, we identified exon 23 to undergo three splicing events- a 63 bp and 30bp alternative acceptor sites and its complete splicing which spans till exon 27. Previous work also suggested that the inclusion of exon 27C might lead to generation of non-

functional isoforms. Splicing in exons 11, 12 and 13 located between the II and III domain in linker region were recorded in this species which are identified as alternative exons in other species, with exon 13 said to specifically express in embryonic stage.

It possible that these transcript variants may play a pivotal role in developmental level and are the tissue specific as evidenced in most eukaryote organisms (Kornblihtt *et al*., 2013). However, this has yet to be examined in this *An.stephensi.* Nonetheless, the data derived in the study provided abundant molecular information on VGSC of *An.stephensi* with comparisons to its orthologues in *An. gambiae* and other representative insect pest, while providing insights into the alternative splicing events of the transmembrane channel which need further investigation.

5.5 Conclusion

Examination of post transcriptional modifications in the VGSC of *An. stephensi* revealed the events of extensive alternative splicing occurring in the channel protein but events of RNA editing is absent. Although a definite association of such events is not found linked to DDT and pyrethroid resistant phenotype, the occurrence might be important from a developmental point of view in insects and would need more exploration.

Chapter 6. CONCLUSION

6.1 Insecticide resistance mechanisms outlined in laboratory DDT and pyrethroid resistant *An. stephensi* colonies

Target-site insensitivity and metabolic resistance were examined in our laboratory selected DDT and pyrethroid resistant *An. stephensi* lines. Independent DDT and deltamethrin lines (DEL) obtained by insecticide pressure for several generations showed a high degree of DDT resistance (LT_{50} at 40 hours and LT_{95} at 155 hours) in the DDT line against 4% DDT, and a 23 fold deltamethrin resistance (LT_{50} at 6 hours and LT_{95} at 42 hours) in DEL against 0.05% deltamethrin. Knockdown resistance mutations, *kdr,* known to confer resistance against DDT and pyrethroids in a plethora of insect species have been observed, but are not likely the key factors responsible for the high resistance in our laboratory colonies. It was observed that the starting cohort of mosquitoes prior to selection pressure had both classic *kdr* mutations L1014F and L1014S. Although in low frequencies (<10%), the fixation of the 1014S in selected DDT- and deltamethrin-resistant lines as well as in control mosquito lines suggests selection of the allele in laboratory conditions with a low fitness cost. Further, metabolic and synergistic assays of the selected DDT-resistant line homozygous for the 1014S *kdr* allele showed high glutathione S-transferase activity. This suggested a role for GST. Since pre-exposure with PBO had no effect on the mortality of mosquitoes exposed to DDT, resistance does not appear to be associated with monooxygenase activity. Acetylcholinesterase activity was, however, noted. The deltamethrin-resistant line, which was also homozygous for L1014S-*kdr* allele, showed significant elevation of monoxygenases and non-specific esterase activity. Synergistic assays with PBO on selected deltamethrin-resistant strains resulted in reversal of susceptibility, confirming the involvement of monooxygenase and esterases in deltamethrin resistance. L1014S may play a minor role, but this would need further investigation. A moderate level of cross-resistance (30% and 35% mortality) against permethrin (type I pyrethroid) and cyfluthrin (type II pyrethroid) respectively was observed in the deltamethrin strain. The elevation of GST observed in both the highly resistant DDT laboratory insecticide strain as well as the deltamethrin strain of *An.stephensi* was further investigated.

6.2 Evidence of tandem co-duplication of epsilon 2 and -4 in the AsGSTe array

The GST epsilon array implicated in DDT resistance in insects was dissected in our laboratory *An. stephensi* strain. Genomic analysis of metabolic mechanism involving GST epsilon array showed evidences of tandem duplication of AsGSTe2 and AsGSTe4 together in GST-epsilon array of the laboratory-maintained DDT-resistance *An. stephensi* colony. The key evidence includes (i) unequal copy number of two AsGSTe2 variants in individuals as evident from DNA sequence chromatogram and variant-specific qPCR, (ii) presence of more than two haplotypes (HAP1, HAP2 and HAP3) in all individuals, (iii) complete heterozygosity for variant alleles tested on two loci, one in the coding region of AsGSTe2 and another in the intergenic region (previously described e2 pseudogene) suggesting that variants are located on different loci in the duplicated gene, and (iv) genomic copy number variations in individuals as detected by qPCR.

The arrangement of the gene duplication and breakpoints in our DDT-resistant strain recorded at least five tandem repeats in a 3.7 kb long unit consisting of AsGSTe1, AsGSTe2, e2-pseudogene, AsGSTe4 and part of AsGSTe5 separated by a 2.7 kb DNA insert of unknown origin. On average, six copies of duplicated genes in DDT-resistant line were evident from qPCR performed on AsGSTe2, whereas very low copy number AsGSTe2 of two were identified in DDT-susceptible strain. Polymorphism in two AsGSTe2 and three AsGSTe4 paralogs due to non-synonymous changes, plus high divergence in intervening e2-pseudogene indicates that gene duplication is not a recent event. Despite the divergence in paralogs, AsGSTe2 and AsGSTe4 expressed as mRNA. The copy number variation at genomic level correlated with RNA expression in limited samples examined, which could positively suggest that gene duplication rather than just overexpression may be the mechanism of DDT resistance in *An. stephensi.*

While qualitative changes driven by a single mutation in this gene resulted in higher metabolic activity (Riveron *et al.,* 2014), several examples of duplication events associated with insecticide resistance have been reported in insect resistant genes (Mouches *et al.,* 1986; Field *et al.,*1988; Campbell *et al.,* 1997, 1998 ; Li *et al.,* 2007; Wondji *et al.,* 2009; Anthony *et al.*, 1998; Remnant *et al.,* 2013; Edi *et al.,* 2014; Kwon

et al., 2010) with most detoxification genes such as esterase and *CYP* undergoing extensive gene amplification to heighten gene dosage. This study suggest that the duplication event of the resistance related AsGSTe2 gene has a possible role in DDT resistance although mutations accompanied with resistant genes reported in insect species are absent in *An. stephensi* to explain fitness consequences.

6.3 Expression and characterisation of recombinant glutathione s transferase epsilon AsGSTe2 and AsGSTe4

Glutathione-S-transferase epsilon 2 as a key factor of metabolic DDT resistance and e4 suspected in pyrethroid resistance was characterized in the DDT resistant *An. stephensi* laboratory colonies. Relative expression profiling of the GST epsilon array in *An. stephensi* using quantitative PCR revealed high expression of AsGSTe2 and AsGSTe4 in DDT resistant strains compared to the susceptible strain. AsGSTe2 implicated in DDT resistance and AsGSTe4, also observed in pyrethroid resistant strains, were characterized at the molecular level for allelic/haplotype variation, which might have a role in resistance. Sequencing and cloning of the two genes revealed polymorphisms in AsGSTe2 with five nonsynonymous changes and a six amino acid repeat Ser- Tyr- Iso- Ser- Ser- Iso giving rise to four AsGSTe2 variants, and in AsGSTe4, residue mutations R3K, S89R and L213R resulted in three variants. Metabolic studies for DDT metabolization of AsGSTe2.1 and AsGSTe2.2, which are the predominant variants of epsilon 2, showed significant DDT dehydrochlorinase activity. *Anopheles stephensi* GSTe4 did not metabolize DDT suggesting no direct role in the first phase DDT metabolism. An analysis of the thermo profile showed the AsGSTe2 variants to be highly unstable at temperatures beyond 20ºC but needs further investigation; on the contrary AsGSTe4 variants exhibit a higher thermostability up to 40ºC compared to *Ae. aegypti* or *An. gambiae.* In- silico analysis and molecular docking of AsGSTe2 showed that none of the variant amino acids lie in the DDT binding site suggesting no association with DDT resistance in this vector.

6.4 Molecular characterisation of the VGSC in *An. stephensi*

The voltage gated insect sodium channel in *An.stephensi* serves as a target for site for DDT and pyrethroids. Knockdown mutations L1014F/S harboured along the channel are known to confer resistance in insect vectors (Ranson *et al*., 2000). While the

channel is essential for signaling in neurons and electrically excitable cells, and have receptor- binding sites for neuro-toxicants (Dong, 2007), gating kinetics of the channel in a few insects have been studied and attributed to post transcriptional events (Song *et al*., 2004; Liu *et al*., 2004; Dong, 2014) due to variants bearing unique functional and pharmacological properties. Splicing events are known in *An. gambiae* (Davies *et al*., 2007) and Drosophila (Hanrahan *et al*., 2000) but are not functionally understood.

The sodium channel of *An. stephensi* harboured the classic *kdr* mutations L1014F and L1014S with two new mutations Q695R and E1235G. The new mutations however were found only in individuals with L1014 genotype and their role in resistance is unknown. F968 and I987 mutations identified are synonymous. The full coding VGSC has 2141 residues spanning 33 exons with a quadrat repeat of thirty nucleotides observed in a few clones in the sequence -5'-CCTTGGACATCAGAGGCTCTCATTGTTGGC-3'lying in the untranslated promoter region. Whether the duplication of nucleotides is at the gDNA has not been ascertained.

Post-transcriptional modification examination showed alternative splicing phenomena, both reported and new events present in *An.stephensi*. Optional exons (2, 5, 12 and 13), mutually exclusive exons (20 and 27), alternative acceptor sites (exons 18, 23a and b and 24), larger skipped exons (12-13, 23-27, 5-19, 1-21) and an intron retention between exons 15 and 16 were identified. However, expression profile of major splice variants showed no significant differences between the variants in the resistant and susceptible strains suggesting no association with resistance. Most of the splice patterns exhibit similarity with other insect VGSC orthologues. RNA editing events at the *kdr*-locus were absent.

6.5 Final Remarks and future perspective

The study confirms suggests the role of GST epsilon 2 in conferring high DDT resistance in a highly resistant DDT laboratory *An.stephensi* strain. We conclude that the overexpression of AsGSTe2 and duplication of the GST epsilon array are linked events in the evolving resistance resulting in the high degree of DDT resistance in this strain. This event has not been reported in other insect species and thus forms an important study to be explored and examined since its occurrence in other species is

likely. The study also confirmed post-transcriptional events of alternative splicing and *kdr* mutations in the VGSC, which is a target site for DDT and pyrethroids in vector while the role of the splice variants and the *kdr* mutations appear to be minor.

Surveillance of malaria transmission and vector population dynamics revealed changes in insecticide susceptibility in an increased number of vector populations as a growing public health concern in India (Kranthi, 2002). DDT has been used extensively in malaria transmission in India owing to its low cost and its past success in curbing malaria epidemicity. Pyrethroids are now the insecticide of choice in bed nets and IRS, and expansion of IRS and LLINS, has since proven to be a cost effective and robust intervention in reducing the burden of disease. The effectiveness of current tools is sadly under an inevitable threat, due to the evolution of emerging insecticide resistance in disease vectors. Development of resistance to DDT and organophosphates is well documented in most insect species (Karunamoorthi *et al*., 2013). Pyrethroid and DDT resistance is a key concern to malaria control programmes for reduction of vector borne disease. Studies on insecticide resistance have shown that insects can exhibit multiple resistance mechanism (Buhler, Pesticidestewardship.org). Identification of the key mechanisms and understanding their operation in insecticide resistance is therefore a fundamental step in curbing the spread of resistance, and this study had its focus on DDT resistance mechanisms in *An. stephensi*.

Better resistance management means development of novel vector control strategies which is made possible only when a full understanding of mosquito biology and its molecular makeup is achieved. The advancement in technology and the wide availability of techniques on the research platform allows for novel genes to be identified and characterized. RNAseq is one such powerful tool with many advantages over gene expression techniques and has been employed in mosquito transcriptome studies by many researchers. Preliminary attempts on whole transcriptome study of DDT resistant and DDT- susceptible *An. stephensi* carried out parallel to this study revealed a series of uncharacterized novel transcripts whose function as a gene remains to be understood. RNAseq analysis validated the over-expression of AsGSTe2-AsGSTe4 and further revealed, a total of 624 significant genes of which 150 genes showing differential expression ($p<0.05$) in DDT resistant strains were identified (unpublished data). Significant variants 630, promoter loci 102 and number of splice

variants 97 were recorded. A series of known resistance implicated genes previously reported such as *cyp*4, ABC transporter, serine protease and hexamarin were found upregulated. Down regulated genes include a few of the alpha esterase family, CYP family including GST epsilon family which is interesting to understand. Such study provides a potential perspective of identifying novel genes and their implicative role in resistance; besides, it paves way for better understanding of known candidate genes whose mechanism of function may be different than that previously known. With an aim to provide a broader picture of the mechanisms operating behind DDT resistance in *An. stephensi* vector, the findings in this study are fundamental to vector management and resistance monitoring programmes. In event of drastic failure of the current efficient and available insecticide classes particularly the pyrethroids, this piece of work may be of interest for informed strategic designing of newer and better tools and insecticide molecules in the very near future.

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APPENDIX

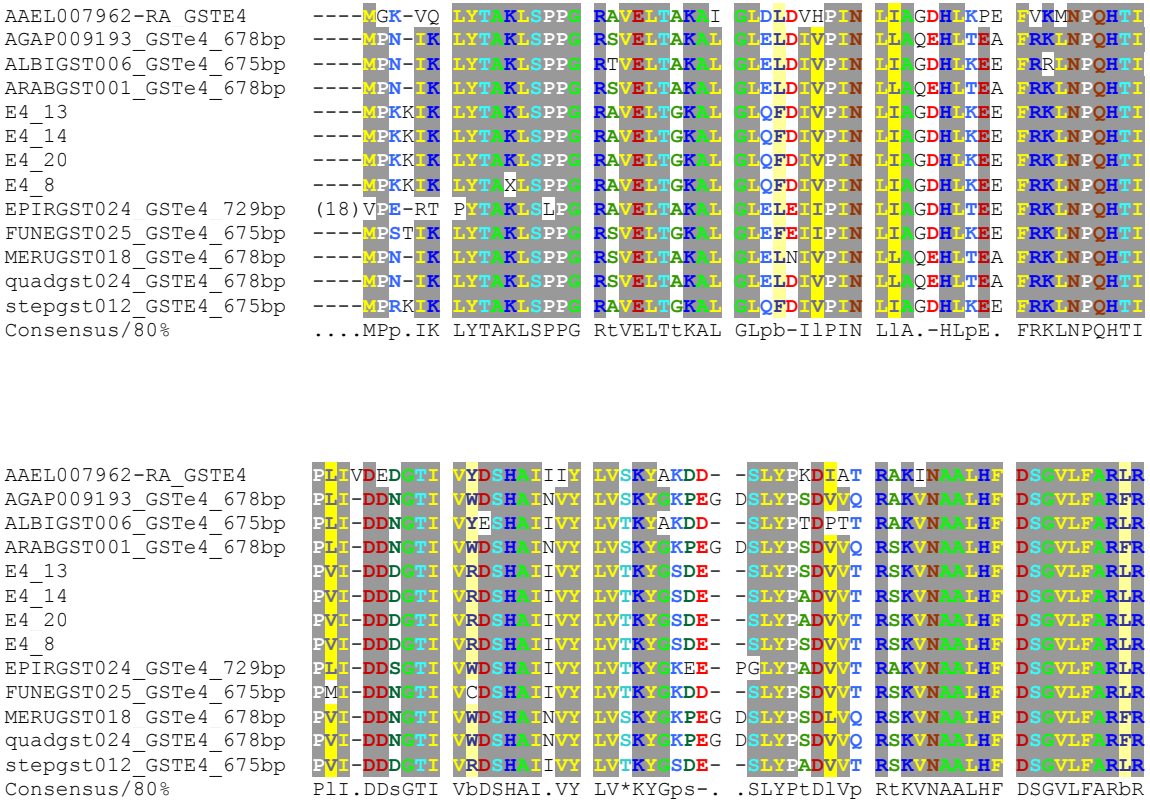
Appendix 3A.] AsGSTe2 nucleotide sequence identity comparison with *An. gambiae* species complex, *An. funestus* and *Aedes aegypti.*

Species	Identity
An.albimanus	78%
An.arabiensis	86%
An.quadriannulatus	86%
An.merus	86%
An.gambiae	86%
An.funestus	86%
An. epiroticus	86%
Aedes aegypti	69%
An. stephensi	100%

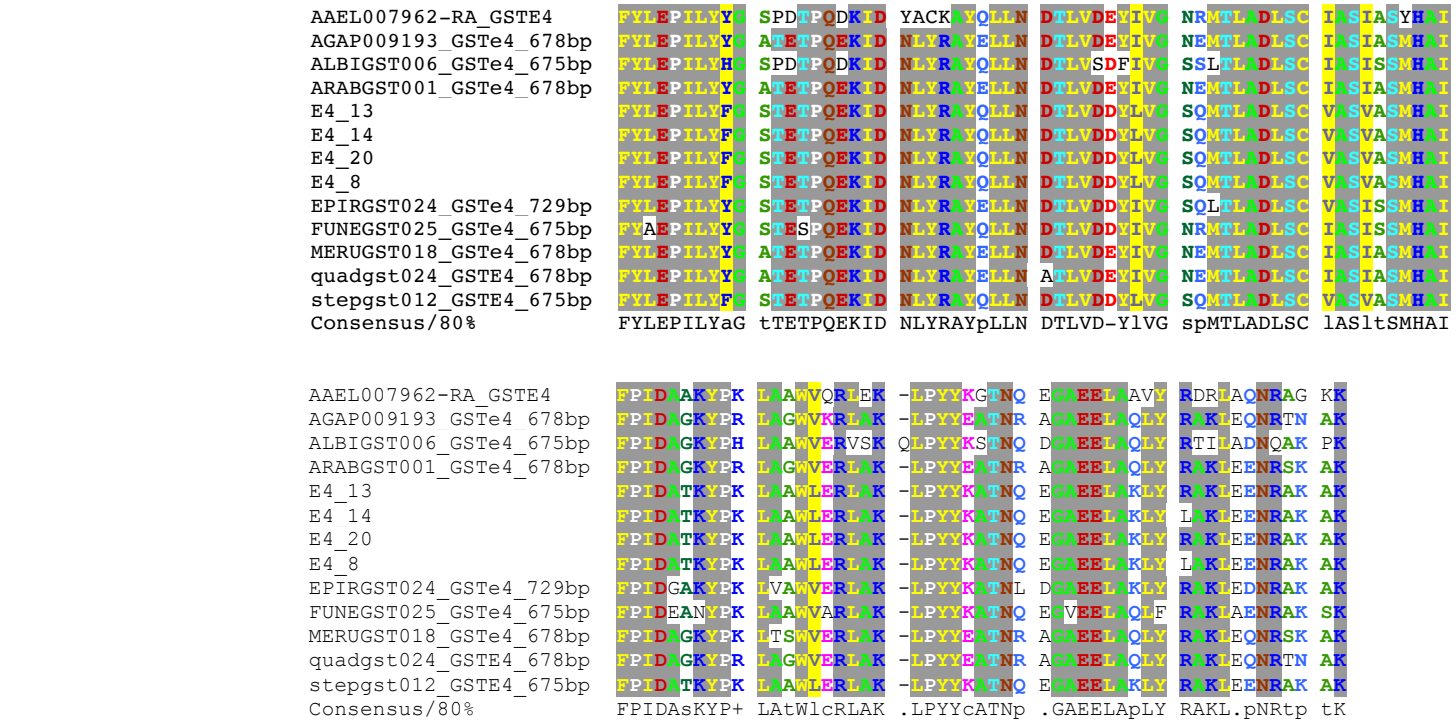
Appendix 3B.] AsGSTe4 nucleotide sequence identity comparison with *An. gambiae* species complex, *An. funestus* and *Aedes aegypti.*

Species	Identity
An. albimanus	76%
An. funestus	83%
An. epiroticus	84%
An. arabiensis	82%
An. quadriannulatus	83%
An. gambiae	82%
An. merus	82%
Aedes aegypti	74%
An.stephensi	100%

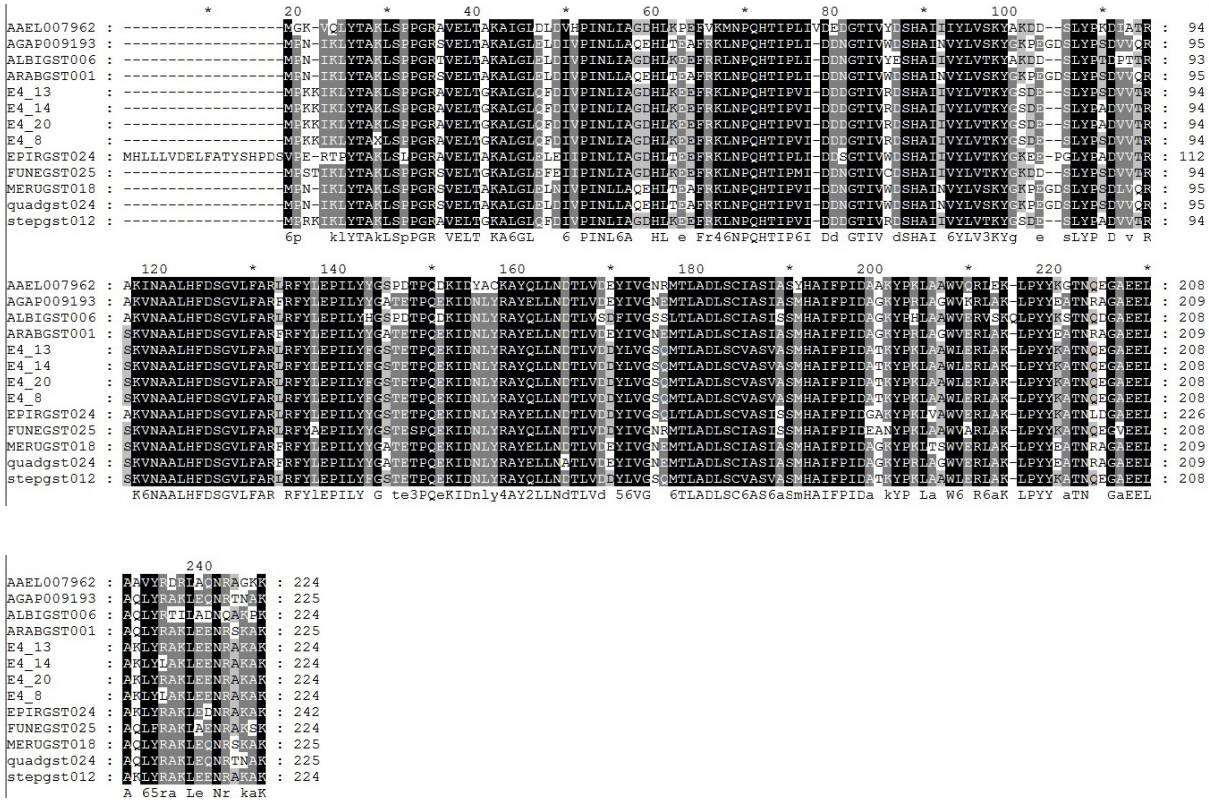
Appendix 3C.] Alignment of *An. stephensi* GSTe4 clones against *An. gambiae complex, An. funestus, Aedes* and *culex.*



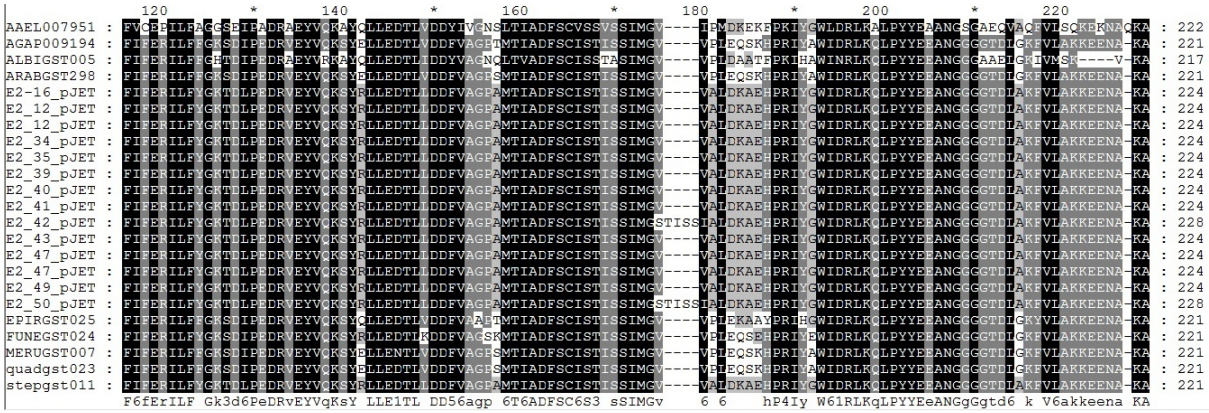
Appendix 3C.] contd..



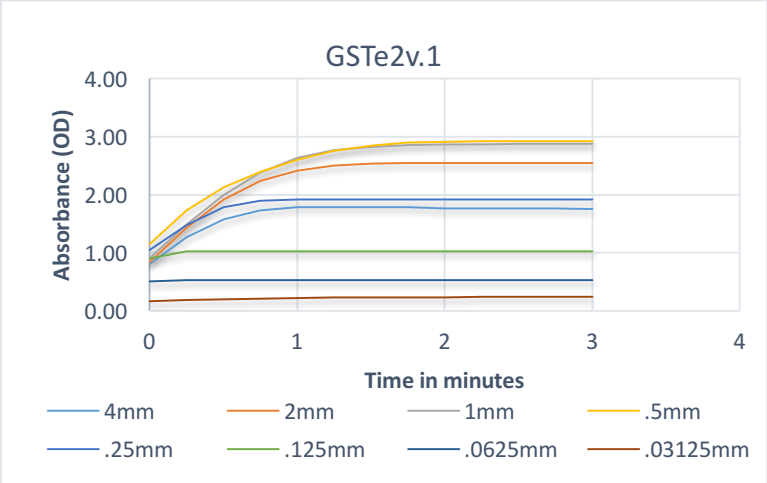
Appendix 3D.] AsGSTe4 clones sequence alignment.

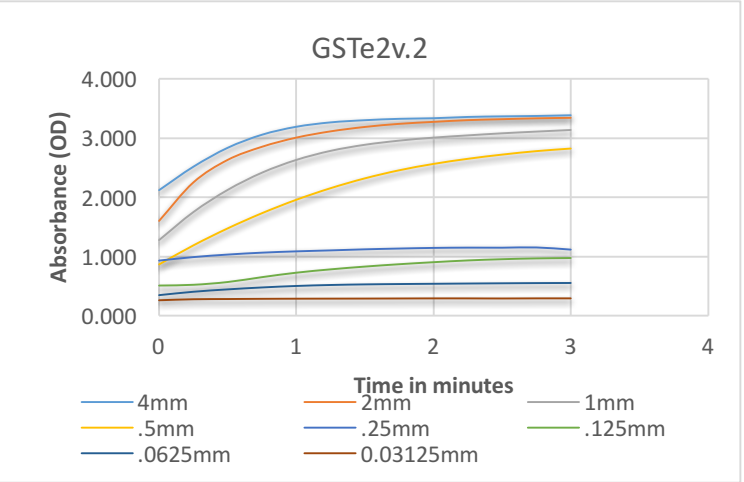


Appendix 3E.] AsGSTe2 clones sequence alignment.



Appendix 4.] Recombinants AsGSTe2.1 and AsGSTe2.2 absorbance change in time with varying concentrations of substrate CDNB (4mM - .03mM).





Appendix 5.] Coding sequence with the deduced amino acids of the voltage gated sodium channel in *An.stephensi.*

```
atgaccgaagactccgattcgatatctgaggaagaacgtagtttgttccgtcctttcact
 M  T  E  D  S  D  S  I  S  E  E  E  R  S  L  F  R  P  F  T
cgtgaatcattacaagctattgaagcacgcatcgccgatgaagaagccaaacaccgagaa
 R  E  S  L  Q  A  I  E  A  R  I  A  D  E  E  A  K  H  R  E
ttggaaagaaagcgagccgaggggggagagtgattttggtcggaagaaaaagaaaaaagaa
 L  E  R  K  R  A  E  G  E  S  D  F  G  R  K  K  K  K  K  E
atacgctacgatgacgaggatgaggatgaaggtccccaaccggaccctacacttgaacag
 I  R  Y  D  D  E  D  E  D  E  G  P  Q  P  D  P  T  L  E  Q
ggtgtaccagtcccagttcgaatgcagggcagcttccctccggagttggcctccacgcct
 G  V  P  V  P  V  R  M  Q  G  S  F  P  P  E  L  A  S  T  P
ctcgaggatattgacggtttttattcgaatcaaaggacattcgtagtgattagtaaagga
 L  E  D  I  D  G  F  Y  S  N  Q  R  T  F  V  V  I  S  K  G
aaagatatttttcgtttctccgccaccaacgcattatacgtactcgatccgtttaaccct
 K  D  I  F  R  F  S  A  T  N  A  L  Y  V  L  D  P  F  N  P
atacgtcgcgtagctatttatatttagtacatccactgttttcacttttttataataacg
 I  R  R  V  A  I  Y  I  L  V  H  P  L  F  S  L  F  I  I  T
accattcttgttaattgtatattgatgattatgcctaccacgccgacagtcgaatctacc
 T  I  L  V  N  C  I  L  M  I  M  P  T  T  P  T  V  E  S  T
gaggtgatattcaccggcatctacacgttcgaatcagctgtaaaagtgatggcgcgaggt
 E  V  I  F  T  G  I  Y  T  F  E  S  A  V  K  V  M  A  R  G
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 F  I  L  Q  P  F  T  Y  L  R  D  A  W  N  W  L  D  F  V  V
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 I  A  L  A  Y  V  T  M  G  I  D  L  G  N  L  A  A  L  R  T
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 F  R  V  L  R  A  L  K  T  V  A  I  V  P  G  L  K  T  I  V
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 G  A  V  I  E  S  V  K  N  L  R  D  V  I  I  L  T  M  F  S
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 L  S  V  F  A  L  M  G  L  Q  I  Y  M  G  V  L  T  Q  K  C
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 I  K  E  F  P  M  D  G  S  W  G  N  L  T  H  E  S  W  E  L
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 F  N  S  N  D  S  N  W  F  Y  S  E  S  G  D  I  P  L  C  G
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 N  S  S  G  A  G  Q  C  D  E  G  Y  I  C  L  Q  G  Y  G  K
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 N  P  N  Y  G  Y  T  S  F  D  T  F  G  W  A  F  L  S  A  F
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 R  L  M  T  Q  D  Y  W  E  N  L  Y  Q  L  V  L  R  S  A  G
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 P  W  H  M  L  F  F  I  V  I  I  F  L  G  S  F  Y  L  V  N
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 L  I  L  A  I  V  A  M  S  Y  D  E  L  Q  K  K  A  E  E  E
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 E  A  A  E  E  E  A  L  R  V  R  E  E  A  A  A  A  K  A  A
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 K  L  E  A  Q  Q  A  A  A  A  A  A  A  N  P  E  I  A  K  S
ccatcggatttctcttgtcacagctatgagttgttcgtcggacaggagaagggcaacgac
 P  S  D  F  S  C  H  S  Y  E  L  F  V  G  Q  E  K  G  N  D
gataacaataaggagaagatgtccatcagaagcgaaggattggagtcggtgagcgaaatc
```

D N N K E K M S I R S E G L E S V S E I
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T R T T A P T A T A A G T A K A R K V S
gcgggggtcgctactgttcaaaaggcttcactttcattacccggttcaccatttaatctt
A G V A T V Q K A S L S L P G S P F N L
cgtagaggatctagaggatcacatcagttcacgatacgtaacggtagaggacgtttcgtt
R R G S R G S H Q F T I R N G R G R F V
ggtgtacctggtagcgatagaaaaccactggtactctcaacatatctcgatgcacaagaa
G V P G S D R K P L V L S T Y L D A Q E
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H L P Y A D D S N A V T P M S E E N G A
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I I V P V Y Y A N L G S R H S S Y T S H
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Q S R I S Y T S H G D L L G G M T K E S
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R L R N R S A R N T N H S I V P P P N A
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T N L S Y A D T N H K G Q R D F D M T Q
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D C T D D A G K I K H N D N P F I E P A
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Q T Q T V V D M K D V M V L N D I I E Q
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A A G R H S R T S D H G V S V Y Y F P T
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E D D D E D G P T F K D K A L E F L M K
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S I M G R T M G A L G N L T F V L C I I
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I F I F A V M G M Q L F G K N Y V D N V
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D R F P D H D L P R W N F T D F M H S F
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M I V F R V L C G E W I E S M W D C M L
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V G D V S C I P F F L A T V V I G N L V
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V L N L F L A L L L S N F G S S S L S A
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P T A D N E T N K I A E A F N R I S R F

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